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(54) Title: CONTINUOUS IN VITRO EVOLUTION

(57) Abstract

The present invention provides a method for the mutation, synthesis and selection of a protein which binds to a target molecule, the method comprising: (a) incubating a replicable mRNA molecule encoding the protein with ribonucleoside triphosphate precursors of RNA and an RNA–directed RNA polymerase, wherein the RNA–directed RNA polymerase replicates the mRNA molecule but introduces mutations thereby generating a population of mutant mRNA molecules; (b) incubating the mutant mRNA molecules from step (a) with a translation system under conditions which result in the synthesis of a population of mutant proteins such that after translation, mutant proteins are linked to their encoding mRNA molecules thereby forming a population of mutant protein/mRNA complexes; (c) selecting one or more mutant protein/mRNA complex(es) by exposing the population of mutant protein/mRNA complexes from step (b) to the target molecule and recovering the mutant protein/mRNA complex(es) bound thereto.

CONTINUOUS IN-VITRO EVOLUTION

FIELD OF THE INVENTION

5   The present invention relates to a method for mutating and selecting target binding proteins in a translation system; and to a polynucleotide construct for use in this method. The method of the present invention may be applied to the generation of molecules of diagnostic and therapeutic utility.

10   **BACKGROUND OF THE INVENTION**

In vitro evolution of proteins involves introducing mutations into known gene sequences to produce a library of mutant sequences, translating the sequences to produce mutant proteins and then selecting mutant proteins with the desired properties. This process has the potential for generating proteins with improved

15   diagnostic and therapeutic utilities. Unfortunately, however, the potential of this process has been limited by deficiencies in methods currently available for mutation and library generation.

For example, the generation of large libraries (eg beyond a library size of 10^{10}) of unique individual genes and their encoded proteins has proven difficult

20   with phage display systems due to limitations in transformation efficiency. A further disadvantage is that methods which utilise phage-display systems (Figure 1) require several sequential steps of mutation, amplification, selection and further mutation (Irving et al.. 1996; Krebber et al., 1995; Stemmer, 1994; Winter et al., 1994).

25   Examples of procedures which have been used to date for affinity maturation of selected proteins. and particularly for the affinity maturation of antibodies, are set out in Table 1. All these methods rely on mutation of genes followed by display and selection of encoded proteins. The particular mutation that is chosen determines the diversity in the resulting gene library. *In vitro* strategies (Table 1)

30   are severely limited by the efficiency in transformation of mutated genes in forming a phage display library. In one *in vivo* cyclical procedure (Table 1 No.1), *E.coli* mutator cells were the vehicle for mutation of recombinant antibody genes. The *E.coli* mutator cells MUTD5-FIT (Irving et al., 1996) which bear a mutated DNAQ gene could be used as the source of the S-30 extracts and therefore allow

35   mutations introduced into DNA during replication as a result of proofreading errors. However, mutation rates are low compared to the required rate. For example, to

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mutate 20 residues with the complete permutation of 20 amino acid requires a library size of 1×10^{26}, an extremely difficult task with currently available phage display methodology.

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Table 1: Affinity maturation strategies

In vivo	Mechanism
1 Mutator cells	Random point mutations
2 SIP-SAP	Co-selection and infection with antibody-antigen pairs
In vitro	
3 DNA shuffling-sexual PCR	Recursive sequence recombination by DNA homology
4 Site directed mutagenesis over selected regions (CDRs)	Oligonucleotide-coded mutations
5 Chain shuffling	Sequential replacement of heavy or light chain domains using phage libraries
6 Error-prone PCR	Polymerase replication errors

1) Irving *et al.* (1996); 2a) Krebber *et al.* (1995); 2b) Duenas and Borrebaeck (1994); 3) Stemmer (1994), Stemmer *et al.* (1995); 4) Yang *et al.* (1995); 5a) Barbas *et al.* (1994); 5b) Winter *et al.* (1994); 6) Gram

10   *et al* (1992).

A selection method which enables the *in vitro* production of complex libraries of mutants which are continuously evolving (mutating) and from which a
15   desired gene may be selected would therefore provide an improved means of affinity maturation (enhancement) of proteins.
In vitro coupled transcription and translation systems
It is well known that a DNA plasmid containing a gene of interest can act as template for transcription when controlled by a control element such as the T7
20   promoter. It is also known that coupled cell -free systems may be used to simultaneously transcribe mRNA and translate the mRNA into peptides (Baranov et al 1993; Kudilicki et al. 1992; Kolosov et al 1992; Morozov et al 1993; Ryabova et al 1989, 1994; Spirin 1990; US 5556769; US5643768; He and Taussig 1997). The

source of cell free systems have generally been *E.coli* S-30 extracts (Mattheakis 1994; Zubay 1973) for prokaryotes and rabbit reticulocyte lysates for eukaryotes. Transcription/translation coupled systems have also been reported (US 5492817; US 5665563; US 5324637) involving prokaryotic cell free extracts (Mattheakis et al

5   1994) and eukaryotic cell free extracts (US 5492817; US 5665563) which have different requirements for effective transcription and translation. In addition, there are requirements for the correct folding of the translated proteins in the prokaryotic and eukaryotic systems. For prokaryotes, protein disulphide isomerase (PDI) and chaperones may be required. Generally in prokaryotes translated proteins are

10 folded after release from the ribosome; however, for correct folding of the newly translated protein attached (tethered) to the ribosome a C terminal anchor may also be necessary. An anchor is a polypeptide spacer that links the newly translated protein domain (s) to the ribosome. The anchor may be a complete protein domain such as an immunoglobulin constant region. In complete contrast to this, in

15 eukaryotic systems the protein is folded as it is synthesised and has no requirement for the addition of prokaryote PDI and chaperones. An anchor may however be beneficial in eukaryotic systems for spacing from, and correct folding of, the newly translated protein attached (tethered) to the ribosome.

Polypeptides synthesised *de novo* in cell-free coupled systems have been
20 displayed on the surface of ribosomes, since for example in the absence of a stop codon the polypeptide is not released from the ribosome. The mRNA ribosome protein complex can be used for selection purposes. This system mimics the process of phage display and selection and is shown in Figure 1. Features required for optimal display on ribosomes have been described by Hanes and Pluckthun

25 (1997). These features include removal of stop codons. However, removal of stop codons results in the addition of protease sensitive sites to the C terminus of the newly translated protein encoded by a ssrA tRNA-like structure. This can be prevented by the inclusion of antisense ssrA oligonucleotides (Keiler et al 1996).

RNA-directed RNA polymerases

30 Qβ bacteriophage is an RNA phage with an efficient replicase (RNA-dependent RNA polymerases are termed replicases or synthetases) for replicating the single-strand genome of coliphage Qβ. Qβ replicase is error-prone and introduces mutations into the RNA calculated *in vivo* at 10^3-10^4 bases. The fidelity of Qβ replicase is low and strongly biased to replicating its template (Rohde et al

35 1995). These teachings indicate that replication over a prolonged period leads to accumulation of mutated strands not suitable for synthesis of a desired protein.

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Both + and - strands serve as templates for replicase; however, for the viral genome the + strand is bound by Qβ replicase and used as the template for the complementary strand (-). In order for RNA replication to occur the replicase requires specific RNA sequence/structural elements which have been well defined
5   (Brown and Gold 1995; Brown and Gold 1996). A reaction containing 0.14 femtograms of recombinant RNA produces 129 nanograms in 30 mins (Lizardi et al 1988).

RNA-directed RNA polymerases are known to replicate RNA exponentially on compatible templates. Compatible templates are RNA molecules with secondary
10   structure such as that seen in MDV-1 RNA (Nishihara,T., et al 1983). In this regard, a vector has been described for constructing amplifiable mRNAs as it possesses the sequences and secondary structure (MDV-1 RNA) required for replication and is replicated *in vitro* in the same manner as Qβ genomic RNA. The MDV-1RNA sequence (a naturally occurring template for Qβ replicase) is one of a number of
15   natural templates compatible with amplification of RNA by Qβ replicase (US-4786600): it possesses tRNA-like structures at its terminus which are similar to structures that occur at the ends of most phage RNAs which increase the stability of embedded mRNA sequences. Linearisation of the plasmid allows it to act as a template for the synthesis of further recombinant MDV-1 RNA. (Lizardi et al 1988).
20   Teachings in the art show that prolonged replication by Qβ replicase of a foreign gene require that it be embedded as RNA within one of the naturally occurring templates such as MDV-1RNA.

SUMMARY OF THE INVENTION

25   The present inventors have now found that RNA directed RNA polymerases introduce mutations into synthesised mRNA molecules during replication in such a manner as to create a library of evolving (mutated) mRNA molecules. These mutated mRNA molecules vary in size due to insertions and deletions as well as point mutations and may be translated *in vitro* such that the corresponding proteins
30   are displayed, for example, on a ternary complex comprising ribosome, mRNA, and mRNA encoded *de novo* synthesised protein. The present inventors have also identified conditions in which a large proportion of proteins generated by the ribosome display process are in a correctly folded, functional form. Furthermore, the present inventors have identified conditions in which phage Qβ replicase can
35   function in eukaryotic coupled transcription/translation systems to amplify RNA templates, incorporating mutations into mRNA.

The mRNA molecules in the preferred transcription/translation system of the present invention are in a continuous cyclic process of replication/mutation/ translation leading to a continuous *in vitro* evolution (CIVE) process.

This CIVE process provides a novel method for *in vitro* evolution of proteins which avoids the limitation of numbers, library size and the time consuming steps inherent in previous affinity maturation processes.

Accordingly. in a first aspect the present invention provides a method for the mutation. synthesis and selection of a protein which binds to a target molecule, the method comprising:

(a)   incubating a replicable mRNA molecule encoding the protein with ribonucleoside triphosphate precursors of RNA and an RNA-directed RNA polymerase. wherein the RNA-directed RNA polymerase replicates the mRNA molecule but introduces mutations thereby generating a population of mutant mRNA molecules:

(b)   incubating the mutant mRNA molecules from step (a) with a translation system under conditions which result in the synthesis of a population of mutant proteins such that after translation, mutant proteins are linked to their encoding mRNA molecules thereby forming a population of mutant protein/mRNA complexes;

(c)   selecting one or more mutant protein/mRNA complex(es) by exposing the population of mutant protein/mRNA complexes from step (b) to the target molecule and recovering the mutant protein/mRNA complex(es) bound thereto: and

(d)   optionally releasing the mRNA molecules from the complex(es).

In a second aspect the present invention provides a method for the mutation. synthesis and selection of a protein which binds to a target molecule which includes:

(b)   incubating the mutant mRNA molecules from step (a) with a translation system under conditions which result in the synthesis of a population of mutant proteins such that after translation, mutant proteins are linked to their encoding mRNA molecules thereby forming a population of mutant protein/mRNA complexes:

(c)   selecting one or more mutant protein/mRNA complex(es) by exposing the population of mutant protein/mRNA complexes from step (b) to the target molecule:

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(d)   repeating steps (a) to (c) one or more times, wherein the replicable mRNA molecule used in step (a) is the mRNA obtained from complex(es) selected in step (c);

(e)   recovering mutant protein complexes bound to the target molecule(s); and

(f)   optionally releasing or recovering the mRNA molecules from the complex(es).

The mRNA from step (d) may be recycled through steps (a) to (c) without purification or isolation from the translation system.

In one embodiment, the mRNA from step (d) is recycled via step (a) while the mRNA is attached to the complex(es) obtained in step (c). In another embodiment, the mRNA is released from the complex(es) obtained in step (c) prior to recycling. The mRNA may be released from the complexes by any suitable mechanism. The mechanism may include raising the temperature of the incubation, or changing the concentration of the compounds used to maintain the complexes intact.

In the context of the present invention, the mRNA may be recycled through steps (a) to (c) by sequential, manual steps. In a preferred embodiment, however, steps (a), (b), (c) and (d) are carried out simultaneously in a single or multiple chambered reaction vessel and the recycling occurs automatically within the vessel.

In the context of the present invention, the mRNA may be recycled through steps (a) to (c) by sequential, manual steps. In a preferred embodiment, however, steps (a), (b), (c) and (d) are carried out simultaneously in a single reaction vessel and the recycling occurs automatically within the vessel.

In another embodiment of the second aspect, the mRNA from step (d) is isolated. The isolated mRNA may be transcribed into cDNA. The resulting cDNA may be cloned into a vector suitable for expression of the encoded protein.

It will be appreciated by those skilled in the art that any suitable complex may be used to link the translated proteins to their encoding mRNAs. For example, the complex may be a mitochondria or other cell organelle suitable for protein display. In a preferred embodiment, the complex is an intact ternary ribosome complex. The ribosome complex preferably comprises at least one ribosome, at least one mRNA molecule and at least one translated polypeptide. This complex allows "ribosome display" of the translated protein. Conditions which are suitable for maintaining ternary ribosome complexes intact following translation are known. For example, deletion or omission of the translation stop codon from the 3' end of the coding sequence results in the maintenance of an intact ternary ribosome

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complex. Sparsomycin or similar compounds may be added to prevent dissociation of the ribosome complex. Maintaining specific concentrations of magnesium salts and lowering GTP levels may also contribute to maintenance of the intact ribosome complex.

5   It will be appreciated by those skilled in the art that preferred embodiments of the present invention involve coupled replication-translation-selection in a recycling batch process, and preferably, in a continuous-flow process (see, for example, Figure 4). Continuous-flow equipment and procedures for translation or transcription-translation are known in the art and can be adapted to the methods of

10   this invention by changing the composition of materials or conditions such as temperature in the reactor. Several systems and their methods of operation are reviewed in Spirin. A.S. (1991), which is incorporated by reference herein. Additional pertinent publications include Spirin et al. (1988); Rattat et al. (1990); Baranov et al. (1989); Ryabova et al. (1989); and Kigawa et al. (1991), all of which

15   are incorporated by reference herein.

By "translation system" we mean a mixture comprising ribosomes, soluble enzymes, transfer RNAs, and an energy regenerating system capable of synthesizing proteins encoded by exogenous mRNA molecules.

In a preferred embodiment, the translation system is a cell-free translation

20   system. Translation according to this embodiment is not limited to any particular cell-free translation system. The system may be derived from a eukaryote, prokaryote or a combination thereof. A crude extract, a partially purified extract or a highly purified extract may be used. Synthetic components may be substituted for natural components. Numerous alternatives are available and are described in

25   the literature. See. for example, Spirin (1990b), which is incorporated by reference herein. Cell free translation systems are also available commercially. In one embodiment of the present invention the cell-free translation system utilises an S-30 extract from *Escherichia coli*. In another embodiment, the cell-free translation system utilises a reticulocyte lysate, preferably a rabbit reticulocyte lysate.

30   The translation system may also comprise compounds which enhance protein folding. To this end. the present inventors have identified conditions in which an increased proportion of proteins produced by the ribosome display process are generated in a folded, functional form. These conditions include the addition of reduced and/or oxidised glutathione to the translation system at a

35   concentration of between 0.1mM and 10mM. Preferably, the translation system comprises oxidised glutathione at a concentration of between 2mM to 5mM.

Preferably, the translation system comprises oxidised glutathione at a concentration of about 2mM and reduced gluthatione at a concentration of between 0.5 mM and 5mM.

In another embodiment of the present invention the translation system consists of or comprises a cell or compartment within a cell. The cell may be derived from a eukaryote or prokaryote.

A number of RNA-directed RNA polymerases (otherwise known as replicases or RNA synthetases) known in the art have been isolated and are suitable for use in the method of the present invention. Examples of these include bacteriophage RNA polymerases, plant virus RNA polymerases and animal virus RNA polymerases. In a preferred embodiment of the present invention, the RNA-directed RNA polymerase introduces mutations into the replicated RNA molecule at a relatively high frequency. preferably at a frequency of at least one mutation in 10^4 bases, more preferably one mutation in 10^3 bases. In a more preferred embodiment the RNA-directed RNA polymerase is selected from the group consisting of Qβ replicase, Hepatitis C RdRp. Vesicular Stomatitis Virus RdRp, Turnip yellow mosaic virus replicase (Deiman et al (1997) and RNA bacteriophage phi 6 RNA-dependent RNA (Ojala and Bamford (1995). Most preferably, the RNA-directed RNA polymerase is Qβ replicase.

The RNA-directed RNA polymerase may be included in the transcription/translation system as a purified protein. Alternatively, the RNA-directed RNA polymerase may be included in the form of a gene template which is expressed simultaneously with step (a), or simultaneously with steps (a), (b) and (c) of the methods of the first or second aspects of the present invention.

In a further preferred embodiment, the RNA-directed RNA polymerase may be fused with or associated with the target molecule. Without wishing to be bound by theory. it is envisaged that in some cases, the binding affinity of the translated protein may be greater than the affinity of the replicase for the mRNA molecule. The binding of the mutant protein/mRNA complex to a target molecule/ RNA-directed RNA polymerase fusion construct would bring the mRNA into the proximity of the RNA-directed RNA polymerase. This may result in preferential further replication and mutation of mRNA molecules of interest.

RNA templates that are replicated by various RNA-dependent RNA polymerases are known in the art and may serve as vectors for producing replicable mRNAs suitable for use in the present invention. Known templates for Qβ replicase include RQ135 RNA. MDV-1 RNA, microvariant RNA, nanovariant RNAs. CT-RNA

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and RQ120 RNA. Qβ RNA, which is also replicated by Qβ replicase, is not preferred, because it has cistrons, and further because the products of those cistrons regulate protein synthesis. Preferred vectors include MDV-1 RNA and RQ135 RNA. The sequences of both are published. See Kramer et al. (1978) (MDV-1 RNA) and

5   Munishkin et al. (1991) J (RQ135), both of which are incorporated by reference herein. They may be made in DNA form by well-known DNA synthesis techniques.

In a preferred embodiment of the first aspect of the present invention, the method further includes the step of transcribing a DNA construct to produce replicable mRNA. DNA encoding the recombinant mRNA can be, but need not be,

10   in the form of a plasmid. It is preferable to use a plasmid and an endonuclease that cleaves the plasmid at or near the end of the sequence that encodes the replicable RNA in which the gene sequence is embedded. Linearization can be performed separately or can be coupled with transcription-replication-translation. Preferably, however. linear DNA is generated by any one of the many available DNA

15   replication reactions and most preferably by the technique of Polymerase Chain Reaction (PCR). For some systems non-linearized plasmids without endonuclease may be preferred. Suitable plasmids may be prepared, for example, by following the teachings of Melton et al (1984a,b) regarding processes for generating RNA by transcription *in vitro* of recombinant plasmids by bacteriophage RNA polymerases,

20   such as T7 RNA polymerase or SP6 RNA polymerase. See, for example, Melton et al. (1984a) and Melton (1984b), which are incorporated by reference herein. It is preferred that transcription begin with the first nucleotide of the sequence encoding the replicable RNA.

In a further preferred embodiment the transcription is carried out

25   simultaneously in a single or multiple chambered reaction vessel, or reactor, with steps (a), (b), (c) of the method according to the first or second aspects of the present invention.

The target molecule may be any compound of interest (or a portion thereof) such as a DNA molecule, a protein, a receptor, a cell surface molecule, a metabolite.

30   an antibody, a hormone a bacterium or a virus.

In a preferred embodiment, the target molecule is bound to a matrix and added to the reaction mixture comprising the complex (displaying translated proteins). The target molecule may be coated, for example, on a matrix such as magnetic beads. The magnetic beads may be Dynabeads. It will be appreciated that

35   the translated proteins will competitively bind to the target molecule. Proteins with higher affinity will preferably displace lower affinity molecules. Thus. the method

of the present invention allows selection of mutant proteins which exhibit improved binding affinities for a target molecule of interest.

The present inventors have also made the surprising findings that minimal sequences derived from naturally occurring replicase templates, such as the MDV-1 template, are sufficient for the binding of Qβ replicase. On the basis of this finding a novel construct suitable for transcription of replicable mRNA has been developed.

Accordingly, in a preferred embodiment of the first or second aspects of the present invention, the method further includes transcribing a DNA construct to produce a replicable mRNA molecule, wherein the DNA construct comprises:

(i)   an untranslated region comprising a control element which promotes transcription of the DNA into mRNA and a ribosome binding site;

(ii)   an open reading frame encoding the protein which binds to the target molecule; and

(iii)   a stem-loop structure situated upstream of the open reading frame.

In a third aspect the present invention provides a DNA construct comprising:

(i)   an untranslated region comprising a control element which promotes transcription of the DNA into mRNA and a ribosome binding site;

(ii)   a cloning site located downstream of the untranslated region; and

(iii)   a replicase binding sequence located upstream of the cloning site.

When used herein the phrase " replicase binding sequence" refers to a polynucleotide sequence which as a "loop-like" secondary structure which is recognised by a replicase (in particular, a replicase holoenzyme). Preferably, the replicase binding sequence does not include a full length RNA template for a replicase molecule. For example, preferably the phrase "replicase binding sequence" does not include full length MDV-1 RNA or RQ135 RNA templates.

In a preferred embodiment, the replicase binding sequence is between 15 to 50 nucleotides in length, more preferably between 20 and 40 nucleotides in length. Preferably, the replicase binding sequence is recognised by Qβ replicase.

In a further preferred embodiment, the sequence of the replicase binding sequence comprises or consists of the sequence: GGGACACGAAAGCCCCAGGAACCUUUCG.

In a further preferred embodiment, a second replicase binding sequence is included downstream of the cloning site.

Any suitable ribosome binding site may be used in the construct of the present invention. Prokaryotic and eukaryotic ribosome binding sequences may be

incorporated depending on whether prokaryotic or eukaryotic systems are being used. A preferred prokaryotic ribosome binding site is that of the MS2 virus.

In a further preferred embodiment, the DNA construct includes a translation initiation sequence. Preferably, the translation initiation sequence is ATG.

5   It will be apparent to those skilled in the art that any gene of interest may be inserted into the cloning site in the DNA construct. In a preferred embodiment the gene(s) of interest is a nucleotide sequence coding for (i) a library of target binding proteins or (ii) a single target binding protein, where the target could include any of protein, DNA, cell surface molecules, receptors, antibodies, hormones, viruses or
10   other molecules or complexes or derivatives thereof.

A nucleotide sequence coding for an anchor domain may be fused 3' in frame with the gene of interest. The anchor domain may be any polypeptide sequence which is long enough to space the protein translated from the gene of interest a sufficient distance from the ribosome to allow correct folding of the molecule and
15   accessibility to its cognate binding partner. Preferably, the polypeptide has a corresponding RNA secondary structure which mimics that of a replicase template. In a preferred embodiment, the polypeptide is an immunoglobulin constant domain. Preferably, the polypeptide is a constant light domain. The constant light domain may be the first constant light region of the mouse antibody 1C3.
20   Preferably, the constant domain is encoded by the sequence shown in Figure 5a. Alternatively, the polypeptide may be the human IgM constant domain. In another embodiment the anchor may be selected from the group consisting of: the octapeptide "FLAG" epitope. DYKDDDDK or a polyhistidine $_8$ tag followed optionally by a translation termination (stop) nucleotide sequence. The translation
25   termination (stop) nucleotide sequence may be TAA or TAG. In some constructs of the present invention. no stop codons are present so as to prevent recognition by release factors and subsequent protein release. In these constructs, the anti-sense ssrA oligonucleotide sequence may be added to prevent addition of a C terminal protease site in the 3' untranslated region that follows.
30   In a fourth aspect the present invention provides a kit for generating a replicable mRNA transcript which includes a DNA construct according to the second aspect of the present invention.

In a preferred embodiment the kit includes at least one other additional component selected from
35   (i)   an RNA-directed RNA polymerase, preferably Qβ replicase, or a DNA
              or RNA template for an RNA-directed RNA polymerase;

    (ii)   a cell free translation system;

    (iii)   a DNA directed RNA polymerase, preferably a bacteriophage
        polymerase;

    (iv)   ribonucleoside triphosphates; and

5   (v)   restriction enzymes.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group

10 of elements or integers.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Affinity maturation cycle for a) phage display and b) ribosome display in the continuos *in-vitro* evolution (CIVE) process.

15 **Figure 2**: Schematic representation of an expression unit containing a gene of interest (nucleotide sequence) for CIVE. The expression unit comprises a gene of interest with upstream ribosome binding site (RBS) and translational initiation site (ATG) along with a transcriptional initiation sequence (T7 promoter). The construct also comprises a downstream spacer sequence.

20 **Figure 3**: Schematic representation of the CIVE method showing the continuous cycling nature of *in vitro* affinity maturation. The method enables the in vitro production of complex libraries of mutants which are continuously evolving (mutating) and from which a desired gene may be selected: the mRNA molecules in the preferred transcription/translation system of the present invention are in a

25 continuous cyclic process of re[plication/mutation/translation leading to continuous *in vitro* evolution (CIVE).

Figure 4: Representation of a reaction vessel suitable for the CIVE process.

Figure 5: Nucleotide sequences of: a) the first constant light region of mouse monoclonal antibody 1C3; b) the third constant heavy region of the human IgM

30 antibody; c) the anti glycophorin (1C3) scFv; d) the anti-HepatitisB surface antigen (4C2) scFv.

Figure 6: DNA sequence of the plasmid pBRT7Qbeta containing a cDNA copy of the Qβ bacteriophage genome.

Figure 7: Schematic representation of the plasmids (a) pGC038CL (containing the

35 anti-glycophorin scFv (1C3) and the mouse constant light region) and (b) pGC_CH (containing the human constant heavy region), which were used for the PCR

synthesis of template used for in vitro transcription and translation. These plasmids were used to supply downstream spacer sequences. In most cases, genes of interest were cloned into SfiI and NotI sites of pGC_CH.

Figure 8: Sequences of RNA fragments that form stem loop structures

5   **Figure 9**: Eukaryotic expression vector pcDNA3.1 for expression of Qβ replicase or Hepatitis C virus RNA dependent RNA polymerase in the rabbit reticulocyte coupled transcription/translation system.

Figure 10: DNA sequence of the Hepatitis C virus RNA dependent RNA polymerase.

10   **Figure 11**: DNA sequences of oligonucleotides used as primers in PCR reactions to generate template DNA for *in vitro* coupled transcription/translation reactions. Nucleotide sequences of oligonucleotides used for both the generation of templates and the recovery of products after panning. Sequences are numbered and are written 5' to 3'.

15   **Figure 12**: Expression of the Qβ replicase in the rabbit reticulocyte coupled transcription/translation system

Figure 13: Effect of Qβ replicase on coupled transcription/translation of anti GlyA 1C3 protein synthesis.

Figure 14: Effect of including Qβ replicase in coupled transcription and translation;

20   Table of mutations in the sequences of selected mutants. This figure shows the positions and type of mutations found in 280 nucleotides of sequence from 6 random clones. These had been recovered from pannings of the anti-GlyA scFv against GlyA coated Dynabeads after transcription and translation either in the absence of Qβ replicase. in the presence of purified Qβ or in the presence of

25   plasmid pCDNAQβ. In the "Mutation Found" column; "None" means that no mutations were found: Mutations are shown in the form AxB where A is the wild type nucleotide. x is the position number within the sequence (as presented in Figure 5c) and B is the mutated nucleotide observed.

Figure 15: Replication of anti glycophorin scFv transcripts by Qβ replicase in the

30   coupled transcription/translation rabbit reticulocyte system: densitometer scanning.

Figure 16: DNA sequence analysis of replication and mutation of anti glycophorin scFv and anti Hepatitis B scFv by Qβ replicase from T7 polymerase transcripts.

Figure 17: Vector containing the Hepatitis C RNA dependent RNA polymerase.

Figure 18: Effect of Hepatitis C RNA dependent RNA polymerase expressed in the

35   coupled transcription/translation system on replication of anti GlyA 1C3 scFv RNA.

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Agarose gel electrophoresis of the RT-PCR products stained with ethidium bromide and scanned.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

5   In a preferred aspect of the present invention, the system for continuous one-step evolution of proteins comprises the following components:

The expression unit

A preferred expression unit for use in the present invention is depicted in Figure 2. This expression unit comprises 3' and 5' untranslated regions with in the

10   5' untranslated region a control element such as the T7 or SP6 promoter to promote transcription of the DNA into mRNA. The consensus DNA sequences are specific for their polymerases; the T7 promoter sequence for T7 RNA polymerase is: TAATACGACTCACTATAGGGAGA. The T7 promoter sequence may act as an RNA dependent RNA polymerase binding sequence (ie. it may act as a binding sequence

15   for Qβ replicase). Preferably, however, the construct includes a stemloop structure for the binding of Qβ replicase, located in the 5' untranslated region 3' to the promoter site. Preferably, a second stemloop structure is included downstream of the coding sequence, preferably about 1 kb 3' of translation termination site of the expression unit. The preferred sequence of the stemloop structure is:

20   GGGACACGAAAGCCCCAGGAACCUUUCG.

The ribosome binding site is the next region downstream of the promoter. Any of several ribosome binding be used in this position. Prokaryotic and eukaryotic ribosome binding sequences may be incorporated depending on whether a eukaryotic or prokaryotic coupled system is being used. One preferred prokaryotic

25   binding site is that of the MS2 virus. The translation initiation sequence ATG is preferably used and codes for the amino acid methionine; this is the start of *in vitro* translation.

The gene (nucleotide sequence) of interest.

It will be apparent to those skilled in the art that the gene of interest can be

30   attached to the untranslated regions by any of the standard genetic techniques. The gene of interest may include any nucleotide sequence with an open reading frame (no stop codons) up to the 3' end of the gene and for the purposes of this invention the end of the anchor (spacing) sequence.

In a preferred embodiment the gene(s) of interest is a nucleotide sequence

35   coding for i) a library of target binding proteins or ii) a single target binding protein, where the target may include any of protein, DNA, cell surface molecules,

receptors, antibodies, hormones, viruses or other molecules or complexes or derivatives thereof. A nucleotide sequence coding for an anchor domain may be fused 3' and in frame with the gene of interest. The anchor domain may be any of a series of polypeptide sequences sufficiently long to space the protein translated

5   from the gene of interest a sufficient distance from the ribosome to allow correct folding of the molecule and accessibility to its cognate binding partner. In a preferred embodiment the anchor is the sequence coding for the octapeptide "FLAG" epitope: DYKDDDDK or any of the human or murine antibody constant domains. Preferably. the anchor is the constant domain from a mouse monoclonal

10 antibody, such as constant domain 1C3 (see Figure 5a). A further preferred anchor is the constant region from a human IgM antibody (see Figure 5b).

The anchor sequence may be followed by a translation termination (stop) nucleotide sequence e.g. TAA or TAG. However, in some constructions it could be envisaged that no stop codons should be present to prevent recognition by release

15 factors and subsequent protein release. In these, the anti-sense ssrA oligonucleotide sequence is added to prevent addition of a C terminal protease site in the 3' untranslated region that follows. The addition of sparsomycin, other similar compounds or a reduction in temperature also prevents release of the ribosome from the mRNA and *de novo* synthesised protein.

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The expression system

Transcription/replication /mutation for the expression unit may be achieved by use of a rabbit reticulocyte lysate system (He and Taussig, 1997)or an *E.coli* S-30 transcription translation mix (Mattheakis et al., 1994; Zubay. 1973). For example a

25 DNA expression unit (detailed above) with a T7 promoter is treated with T7 RNA polymerase according to the manufacturers instructions. The resulting RNA library reflects the diversity of the encoded genes. RNA dependent-RNA polymerases added for replication and mutation can be supplied either as purified enzyme or alternatively encoded as a distinct expression unit in a plasmid under control of a

30 promoter such as T7 or SP6. The preferred enzyme is Qβ replicase although any enzyme with similar characteristics may be used. This step provides the increase in complexity of the library through mutation by the Qβ replicase. For mRNA synthesis in eukaryotic cells the mRNA is preferably capped which is achieved by adding an excess of diguanosine triphosphate; however, the rabbit reticulocyte

35 system from the commercial suppliers Promega and Novagen have components in the system to make the addition of capping compounds unnecessary. The

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transcription/translation mix or coupled system may be extracted from any cell, those most commonly used are wheat germ, mammalian cells such as HeLa cells E.coli and rabbit reticulocytes. The coupled transcription translation system may be extracted from the *E.coli* mutator cells MUTD5-FIT (Irving et al., 1996) which

5   bear a mutated DNAQ gene and therefore allow further random mutations introduced into DNA during replication as a result of proofreading errors. One preferred transcription/translation mix is the rabbit reticulocyte lysate. Addition of GSSG to the coupled system enhances correct folding of displayed proteins and therefore enhances subsequent binding and selection to counter-receptors or

10   antigens.

Mutation by Qβ replicase

The Qβ replicase is included in the system for the replication and production of high levels of mRNA incorporating random mutations (see Figure 3). Multiple copies of a single-stranded RNA template is replicated with mutations by the Qβ

15   replicase into its single stranded complement; however, both strands are equally efficient as template under isothermal conditions.

Teaching in the art indicates that the complex and stable secondary and tertiary structures present in full length RNA from phages such as Qβ limit the access of ribosomes to the protein initiation sites. However, we have found that

20   smaller RNA sequences are suitable for binding of replicases and therefore may be used instead of full length templates. Preferred sequences are small synthetic RNA sequences known as pseudoknots (Brown and Gold 1995;1996) which are compatible with amplification by Qβ replicase. In the context of the present invention, the use of pseudoknots can overcome the problems of ribosome access to

25   the protein initiation sites whilst maintaining the binding sites necessary and sufficient for the Qβ replicase amplification of the RNA and sequences fused thereto.

Translation and Ribosome display

Several *in vitro* translation methods are known which may be either

30   eukaryotic such as rabbit reticulocyte lysate and wheatgerm, or prokaryotic such as E.coli. These are available commercially or can be generated by well known published methods. Translation of the mutated mRNAs produces a library of protein molecules, preferably attached to the ribosome in a ternary ribosome complex which includes the encoding specific mRNA for the de novo synthesised

35   protein (Mattheakis et al., 1994). Several methods are known to prevent dissociation of the mRNA from the protein and ribosome. For example,

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sparsomycin or similar compounds may be added; sparsomycin inhibits peptidyl transferase in all organisms studied and may act by formation of an inert complex with the ribosome (Ghee et al., 1996). Maintaining high concentrations of magnesium salts and lowering GTP levels may also contribute to maintaining the
5   ribosome/mRNA/protein complex; in conjunction with the structure of the expression unit detailed above. A preferred means to maintain the ternary ribosome complex is the omission of the translation stop codon at end of the coding sequence.

In addition. there are preferred requirements for the correct folding of the
10   molecules in the two systems. For prokaryotes protein disulphide isomerase (PDI) and chaperones may be used as well as a C terminal anchor domain to ensure the correct folding. The latter is required as prokaryotic proteins are released from the ribosomes prior to folding (Ryabova et al.. 1997) and therefore in *situations* in which the peptide is anchored to the ribosome the entire protein needs to be spaced
15   from the ribosome. In contrast to this in eukaryotic systems the protein is folded as it is synthesised and has no requirement for the prokaryote PDI and chaperones to be added; however. we have found that addition of a specific range of GSSG concentrations is beneficial to the library selection by the enhanced display of correctly folded proteins on the ternary ribosome complexes.

20

Selection and competitive binding

Successive rounds of RNA replication produce libraries of RNA molecules which on translation produce libraries of proteins. A target molecule-bound matrix (for example antigen-coated Dynabeads) may be added to the reaction to capture
25   ternary ribosome complexes. The individual members in the library compete for the antigen immobilised on the matrix (Dynabeads). Molecules with a higher affinity will displace lower affinity molecules. At the completion of the process the complexes [mRNA/ribosomes/protein] attached to matrix (Dynabeads) may be recovered, cDNA may be synthesised from the mRNA in the complex and cloned
30   into a vector suitable for high-level expression from the encoded gene sequence.

A recycling flow system (Spirin et al.. 1988) may be applied to this Continuous in vitro Evolution (CIVE) system using a thermostated chamber to ensure supply of substrates (including ribosomes) and reagents and removal of non-essential products. All processes of CIVE may take place within this chamber
35   including: coupled transcription and translation, mutating replication, display of the *de novo* synthesised protein on the surface of the ternary ribosome complex and

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competitive binding of the displayed proteins on the ternary ribosome complex to antigen to select those with the highest affinity binding (Figure 4). The unbound reagents, products and displayed proteins are removed by flushing with washing buffer and the bound ternary ribosome complexes are dissociated by increasing the

5   temperature and omitting the magnesium from the buffer. This is followed with the addition of all the reagents necessary to carry out all the above steps except the washing buffer steps. Methods are available to prevent dissociation of the mRNA from the protein and ribosome such as the addition of sparsomycin or similar compounds. maintaining specific concentrations of magnesium salts and lowering

10   GTP levels may also contribute to maintaining the ribosome/mRNA/protein complex as well as reducing the reaction temperature or omitting translational stop codons. By using vessels whose temperatures are controlled combined with a continuous flow capability, mRNAs from selected ribosomes may be dissociated from the ribosomes and further replicated, mutated and translated as the concentration of

15   reagents important for the maintenance of the ribosome/mRNA/protein complex such as sparsomycin. Mg etc are varied. Figure 4 depicts the design of a such a device.

        The present invention will now be more fully described with reference to the following non-limiting Examples.

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Example 1
Recombinant Qβ replicase: Expression and purification
Cloning and expression

        The Qβ replicase coding sequence was amplified by PCR from the plasmid

25   pBRT7Qβ, a pBR322 based construction (briefly described in Barrera et al., 1993) that was designed to allow the preparation of infectious RNA by transcription using T7 RNA polymerase *in vitro*; being a cDNA copy of the RNA genome of phage Qβ. The sequence of pBRT7Qβ is shown in Figure 6. Nucleotide no. 1 is the first nucleotide of the Qβ replicase sense strand. The oligonucleotides used as primers

30   to amplify the Qβ replicase encoded sites for restriction enzyme digestion by the enzymes *Eco*RI and *Not* I and the sequences are shown in Figure 11.

        The PCR products were purified using any one of the commercial products available for this purpose (for example Bresatec). The purified DNA was cloned into the *Eco*RI and *Not*I sites of the vector pGC (Figure 7a) using standard molecular

35   biology techniques. The vector pGC and expression of recombinant protein therefrom has been described in the literature and is incorporated herein by

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reference (Coia et al., 1996). The process of the PCR amplification and cloning of the Qβ replicase gene into vectors and transformation into *E.coli* for expression of the enzyme will be known to those skilled in the art as will be the expression of the Qβ replicase gene in pGC which was induced by adding 1mM

5   ispropylthiogalatoside (IPTG) to the culture medium.

Expression and purification of the Qβ replicase gene in the pBR322 based vector with the promoter PL was performed as detailed below. The rep14 Billeter strain was supplied by Christof Biebricher, Max Planck, Gottingen. The *E.coli* strain was grown in a 20 l fermentor in 2% nutrient broth, 1.5% yeast extract, 0.5%

10   NaCl, 0.4% glycerol, 100mg/l ampicillin with good aeration at 30°C to an optical density of 2 (660nM). After raising the temperature to 37°C, aeration was continued for 5 h. The cells were chilled on ice and harvested by centrifugation (yielding about 180 g wet cell mass).

15   Purification of Qβ replicase

Buffer A: 0.05M Tris.HCl-buffer (pH 7.8), 1mM mercaptoethanol, 20% v/v glycerol, 100 mg/l ampicillin.

Buffer B: 0.05M HEPES.Na-buffer (pH 7.0), 1mM mercaptoethanol, 20% v/v glycerol.

20

50g harvested *E.coli* were homogenized with 100ml. 0.05M Tris.HCl buffer (pH 8.7) 1mM mercaptoethanol in a high-speed blender. Lysozyme and EDTA were added to final concentrations of 100μg/ml and 0.5mM, respectively, and the solution was gently stirred at 0°C for 30 min. 12ml 8% Na deoxycholate, 0.24ml

25   phenylsulfonylfluoride (20mg/ml in propanol-2), 0.15ml Bacitracine (10mg/ml), 0.15ml 0.1M benzamidine, 3.3ml 10% Triton-X-100 were added and the solution adjusted with MgCl$_2$ to 10mM final concentration. The high viscosity was reduced by blending at high speed. Solid NaCl was added to a final concentration of 0.5M and 4.8ml 0.3% polyethyleneimine (pH 8) was added with stirring. After stirring

30   for 20 min at 0°C the suspension was centrifuged for 30 min at 10,000 rpm (GSA rotor). After dilution of the supernatant with 5 volumes Tris.HCl buffer (pH 8.7) 1mM mercaptoethanol, 100ml DEAE cellulose slurry (Whatman DE52, equilibrated with buffer A) was added and slowly stirred at 0°C for 20 min. After 40 min incubation without stirring, the supernatant was decanted from the sediment and

35   discarded. The sediment was suspended in buffer A, poured into a glass column of 1cm diameter, washed with 400ml Tris.HCl buffer (pH 8.7) 1mM mercaptoethanol.

and eluted with 250ml buffer A + 180mM NaCl; fractions were collected. The fractions were assayed for the presence of Qβ replicase using the following binding assay.

5   Enzyme location assay: binding of biotinylated RNA to Qβ replicase
        This is a non-radioactive assay developed to detect replication enzymes which relies on biotin-labelled RNA bound to enzyme being retained on positively charged membranes: whereas, free biotin-labelled RNA under the same conditions is not retained on the membrane. DNA and RNA were labelled with psoralen-biotin
10   (Ambion) according to the manufacturers instructions. The labelled RNA was then added to the column eluate (sample fractions) as indicated in the assay below to detect the location of Qβ replicase.   - --

     The following was mixed in an Eppendorf tube:
15   10µl column eluate fractions
     10µl 0.5M Tris HCl (pH 7.4) containing 120mM $MgCl_2$
     10µl 2mM ATP
     10µl 5mM ATP
     10µl ~100ng/ml psoralen-biotin labelled probe RNA
20   50µl water
     The reaction mix was incubated at 37°C for 1min.

     The reaction mixtures were dot blotted onto nylon membrane, e.g.hybond N, (only RNA or DNA bound to the enzyme Qb replicase will be retained on the membrane),
25   washed with the 50mM Tris HCl pH 7.4 containing 12mM $MgCl_2$, UV cross linked onto the nylon membrane in the Stratalinker on the automatic setting. The BrightStar Biodetect kit was used for the detection of the biotinylated nucleic acid attached to the nylon membrane. Figure 12 shows the assay of the eluted fractions from the DE52 column.
30   The active fractions were pooled, diluted with one volume buffer A and applied to a 35ml column of DEAE-Sepharose FF, equilibrated to buffer A + 0.1M NaCl. The enzyme was eluted with a linear gradient of 0.1-0.4M NaCl in buffer A. The active fractions were pooled, the enzyme precipitated by addition of solid $(NH_4)_2SO_4$ (39g / 100ml solution), collected by centrifugation and dissolved in 4ml
35   buffer B.

The enzyme was diluted until the conductivity was less than that of buffer B + 0.2M NaCl and applied to a 100ml column of Fractogel EMD SO3 equilibrated with buffer B, and eluted with a linear gradient (2 times 500ml) of 0.2--0.8~M NaCl in buffer B. The active peaks, eluting at about 0.65M NaCl, were pooled,

5   precipitated with solid $(NH_4)_2SO_4$ (39g / 100ml solution), collected by centrifigation, and dissolved in 10ml buffer A + 50% glycerol. The solution was stored at -80°C.

The following steps were performed at small scale according to Sumper & Luce (1975). 4 mg Qβ replicase were applied to a 1.6 x 14.5cm column of QAE-

10   Sephadex~A-25 equilibrated with buffer A (diluted or dialysed to remove salt), and eluted with a 2x200ml gradient of 0.05--0.25M NaCl in buffer A. The two clearly separated peaks of core and holoenzyme were pooled, diluted 1:1 with buffer A and applied to QAE-Sephadex columns. 2ml for core, 6ml for holo replicase, respectively. washed with buffer A + 50% glycerol, and the replicase was eluted in

15   concentrated form with buffer A + 50% glycerol + 0.2 M $(NH_4)_2SO_4$. The active fractions were stored at -80°C. Care was taken to avoid contamination of the equipment with RNA.

Example 2

20   **Cloning of Qβ replicase into the eukaryotic expression vector pCDNA3.1**

Qβ replicase coding sequence was cloned into the eukaryotic expression vector pCDNA 3.1 (Figure 9) to produce the vector named pCDNAQB. This vector was used for the expression of Qβ replicase *in situ* in the coupled transcription/translation system and concomitant replication/mutation of target

25   RNA. Sequence of oligonucleotides used as primers in PCR amplification of Qβ replicase for cloning into the *Eco*RI and *Not*I restriction sites in the eukaryotic expression vector pCDNA3.1 were:

5352 5'TCTGCAGAATTCGCCGCCACCATGTCTAAGACAGCATCTTCG

5350 5'TTTATAATCTGCGGCCGCTTACGCCTCGTGTAGAGACGC

30   The coding sequence for the Qβ replicase b subunit was cloned into the pCDNA3.1 by standard molecular biology techniques (Sambrook et al., 1989). The cloned sequence was confirmed by DNA sequence analysis. Expression of the Qβ replicase in the rabbit reticulocyte coupled transcription/translation system was followed by the detection of biotinylated lysine (TRANSCEND, Promega)

35   incorporated into the de novo synthesised Qβ replicase in the standard transcription/translation reaction as suggested by the commercial suppliers of the

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coupled trancription translation kits (Promega and Novagen) and the supplier of
Transcend (Promega). At the completion of the incubation step of the coupled
reaction, 20μl of the reaction was heated to 90°C with 2ml of 10X SDS sample
buffer and the samples subjected to SDS polyacrylamide gel electrophoresis (SDS-
5   PAGE). This was followed by Western blotting and the *de novo* synthesised
biotinylated Qβ replicase bands detected with TRANSCEND kit detection reagents.
The results of this expression are shown in the gel scans of Figure 12 where it can
be seen that Qβ replicase has been synthesised shown by the biotinylated band at
the correct size on the gel.

10   We then undertook coupled Transcription/translation reactions with the 1C3
template (example 3) but also expressing the Qβ replicase from pcDNA3.1 in the
same reaction. The Qβ replicase synthesised *in-situ* from the expression vector
pCDNAQβ resulted in the increased synthesis of the 1C3 scFv in the coupled
system in the presence of 0.5mM manganese chloride; measured by incorporation
15   of biotinylated lysine (Figure 12b) as described above. The presence of the
manganese chloride has previously been shown to relax the dependence of the Qβ
replication activity on transcription/translation factors.

Example 3
20   **Construction by PCR of DNA templates for transcription.**
DNA sequences were amplified by standard and well-described techniques
(Polymerase Chain Reaction [PCR] with specifically designed oligonucleotide
primers, splice overlap extension, restriction enzyme digests etc) using either *Taq*.
Tth, *Tfl*, *Pwo* or *Pfu* polymerase according to the supplier's instructions using either
25   an FTS-1 thermal sequencer (Corbett Research), a PE2400 (PerkinElmer) or a
Robocylcer gradient 96 (Stratagene). A list of oligonucleotide primers used is given
in Figure 11. Products were gel purified using BresaClean (Bresa) or used directly
in coupled transcription and translation reactions.
DNA sequences were amplified from starting templates which had been
30   cloned into either vector pGC038CL (Figure 7a) or pGC_CH (Figure 7b) which
provided an extension to the 3' terminus of the construct. This extension was
either a constant region from a mouse monoclonal (1C3; Sequence Figure 5a) or a
constant region from a human IgM antibody (Sequence Fig. 5b). Forward (sense)
primers (N5266 for the anti-GlyA scFv; N5517 or N5384, N5344 and N5343 for the
35   anti-HepB scFv) used for amplification provided a transcriptional initiation site as
well as a translational initiation site and ribosome binding site. Reverse (anitsense)

primers (N5267 for the mouse constant region; N5385 for the human constant region) did not contain stop codons which allows the mRNA-ribosome-protein complex to remain associated. Both forward and reverse primers provided restriction enzyme sites (specifically SfiI and NotI, respectively) which enabled

5   cloning of generated fragments.

Any of several promoter sequences for DNA dependent RNA polymerase can be used to direct transcription; however, the following sequences were the two preferred (these include translational initiation sequences; see below):

a) GCGCGAATACGACTCACTATAGAGGGACAAACCGCCATGGCC

10   b) GCAGCTAATACGACTCACTATAGGAACAGACCACCATGGCC

These sequences have directed transcription of T7 DNA dependent RNA polymerase to produce RNA transcripts in two alternative formats of coupled transcription/translation systems.

Sequences encoding ribosome binding sites are known and have been

15   included in the template upstream of the any one of the sequences of molecules of interest for ribosome display encoding either the scFv binding to glycophorin (1C3; Figure 5c) or the scFv binding hepatitis B surface antigen (4C2; Figure 5d). The same sequences have been included in the template upstream of any other sequences of interest for ribosome display (eg CTLA-4-based library sequences).

20

Example 4

Coupled transcription/translation and ribosome display in rabbit reticulocyte lysate cell free system.

Transcription and translation was carried out in Siliconized Rnase-free 0.5ml

25   tubes (Ambion) using the TNT T7 coupled transcription/translation system (Promega) containing 0.5mM magnesium acetate, 0.02mM methionine and 3mM oxidized glutathione (GSSG) (see Example 6, below) and the mixture was incubated at 60°C for 90 min. In some reactions up to 10mM reduced glutathione was also added. In reactions containing $Q\beta$ polymerase, the mixture also contained

30   manganese chloride to a final concentration of 0.5mM. After transcription and translation, the mixture was diluted with PBS and treated with DNaseI to remove any remaining starting DNA template. This was achieved by the addition of 40mM Tris (pH 7.5), 6mM $MgCl_2$ 10mM NaCl and DNase I (Promega), followed by incubation at 30°C for a further 20min.

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Example 5

Selection of ribosome ternary complex displayed proteins against antigens using Dynabeads

Tosylactivated Magnetic beads (Dynal) were coupled to GlycophorinA (GlyA;
5 Sigma), HepatitisB Surface Antigen (HepB SA; BiosPacific, Emeryville, CA USA) or
bovine serum albumin (BSA; Sigma) according to manufacturer's instructions.
Where Streptavidin magnetic beads were used, these were coupled (according to
manufacturer's instructions) to antigens (as shown above) which had been
biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce) according to
10 manufacturer's instructions.

In order to select specifically binding mRNA-ribosome-protein complexes, 2-
3μl of antigen coupled (tosylactivated or streptavidin-coated) magnetic beads were
added to the final translation mixture and placed on a plate shaker (Raytek
Instruments) at room temperature for 90 min with gentle shaking to prevent settling
15 of the beads. The beads were recovered using a magnetic particle concentrator
(Dynal) and these were washed three times with cold phosphate buffered saline
(PBS) pH 7.4 containing 1% Tween and 5mM magnesium acetate. The beads were
then washed once with cold sterile water and finally resuspended in 10μl of sterile
water.

20 For the synthesis of cDNA from selected complexes, 2μl of the final bead
suspension was used in an RT-PCR reaction using either the Access RT-PCR system
(Promega) or the Titan One-tube RT-PCR system (Boehringer Mannheim) according
to manufacturer's instructions. The primers used for this reaction included the
original forward (sense) primer (used to generate the starting template DNA
25 primers; N5266 for the anti-GlyA scFv; N5517 or N5384, N5344 and N5343 for the
anti-HepB scFv) and a negative (antisense) primer which was upstream of the
original primer (N5268 and N5269 for mouse constant region constructs; N5386 and
N5387 for human constant region constructs). In some cases, shorter primers
(N5941 and N5942 for the anti-GlyA scFv-constant light region construct) were used
30 to recover panned RNA templates.

For further cycles of selection, this DNA was gel purified (in some cases,
simply diluted) and incorporated into in a further PCR using the forward and
reverse primers which had been present in the original PCR to generate the starting
DNA template. This new template could then be used in further selections as
35 described above since it contained the appropriate initiation sites and is of the same
length as the template in the first selection.

In order to show that the method described above can be used to select
specific molecules. a single chain Fv (scFv) fused to a mouse constant light chain
region which specifically binds to GlyA was amplified using primers which would
allow the addition of a T7 transcriptional initiation site and a ribosome binding site.
5   This template (T7-scFv) was used in a coupled transcription/translation reaction as
described above and then split into three and mixed with either HepB SA, GlyA or
BSA coupled magnetic beads. The beads were washed (as described above) and
recovered mRNA-ribosome-protein complexes were used to synthesize cDNA. The
results of this experiment showed the presence of a product of the correct size in
10   each lane. The non-specific binding observed in the HepB SA and BSA lanes is
probably due to aggregation of products synthesized during translation. It has been
observed by others that only a proportion of-products synthesized using the
reticulocyte lysate are in a properly folded and active form. This problem was
addressed in Example 6 below.
15   The GlyA specific product from this experiment was gel purified and re-
amplified by PCR in order to synthesize more template for a further round of
selection. A second round of panning showed predominantly a specific product in
the sample probed with GlyA coupled magnetic beads. This showed that by the
second round of selection, the products recovered were specific for GlyA.
20

Example 6
Effect of adding oxidized and/or reduced glutathione
In a attempt to induce a higher proportion of correctly folded products
during *in vitro* transcription and translation, various concentrations of either
25   reduced or oxidized glutathione were added to the reaction mixture. The template
used for these reactions was the anti-GlyA T7-scFv (as described above) and
selections were performed using GlyA coupled magnetic beads. This experiment
showed that the amount of recovered product increased with increasing
concentrations of oxidized glutathione up to 5mM. A further increase to 10mM
30   had a detrimental effect on the yield of recovered product. A concentration of
around 2mM oxidized glutathione was included in most transcriptions and
translations.
Later results revealed that a further addition of 5mM and 10mM reduced
glutathione to the reaction already containing 2mM oxidized glutathione showed
35   that the addition of 5mM glutathione appeared to allow better folding of the
displayed anti-GlyA scFv leading to an increased amount of recovered product from

the GlyA panning over the control pannings. Further decreasing the concentration
of reduced glutathione to to 0.5mM showed similar effects.

Example 7

5   **Display of mutation v-domain (CTLA-4) library on ribosomes**

In order to show that ribosome display could be used to select binding
elements from a polypeptide library, a library of CTLA4 mutants was ligated into
plasmid pGC_CH (Figure 7b) which allowed the addition of a constant heavy
domain and this library was then amplified by PCR using primers N5659 and N5385

10   (Figure 11). Primer N5659 was used to add the necessary upstream transcriptional
and translational initiation sequences. This PCR DNA was then used as template
for transcription and translation in a coupled cell free translation system using the
methods described in Example 4. To demonstrate binding of mutant CTLA
ribosome complexes. panning was performed using Hepatitis B surface antigen

15   (HBSA). GlycophorinA (GlyA) and Bovine Serum Albumin (BSA) coated
Dynabeads. RNA attached to bound complexes was then recovered by RT-PCR.
The methods used for panning, selection and recovery was as described previously
(Example 5).

Products corresponding approximately to the size of CTLA4 based mutants

20   were recovered and showed that the CTLA4 library contained DNA encoding
proteins which specifically bind HBSA, GlyA and BSA. These products were
cloned into the vector pGC_CH (Figure 7b) for DNA sequencing and expression of
soluble products. Sequencing using standard methods (BigDye Terminator Cycle
Sequencing; PE Applied Biosystems CA) showed that CTLA4-based specific inserts

25   were present. Furthermore, expression analyses using ELISA showed that
specifically reactive proteins were being expressed by the recombinant cultures. In
these assays, recombinants which had been isolated by panning using GlyA-coated
Dynabeads and screened by ELISA using GlyA-coated plates, gave stronger signals
than similarly tested recombinants which had been isolated by panning using BSA-

30   coated Dynabeads.

Example 8

Effect of including Qβ replicase in coupled transcription and translation.

In a attempt to both increase the yield of products, and increase the rate of

35   mutagenesis in products during in vitro translation, Qβ replicase (in either of two
forms) were added to the reaction mixture. The replicase was included as either a

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purified Qβ replicase protein or as a gene template under the control of a T7 transcriptional promoter (pCDNAQβ) which could be simultaneously synthesized during the coupled transcription/translation reaction. The template used for this reaction was again the anti-GlyA T7-scFv (as described above) and selections were

5   performed using GlyA coupled magnetic beads These experiments showed that the amount of recovered GlyA reactive product increased (over the no Qβ replicase control) with the addition of purified Qβ replicase and, to a lesser extent, with the addition of Qβ replicase-encoding genomic template (pCDNAQβ).

In order to determine whether mutations had been inserted into the scFv

10   sequence, the main product from each lane was gel isolated and purified. The DNA was digested with *Sfi*I and *Not*I and ligated into similarly digested pGC vector and transformed into *E. coli* using standard protocols. DNA was isolated from recombinants from each series and six random clones from each series were subjected to DNA sequencing using standard methods (BigDye Terminator Cycle

15   Sequencing; PE Applied Biosystems CA). Approximately 280 bases were sequenced from each clone and Figure 14 shows the number and the position of mutations in these sequences. This experiment showed the introduction of an increased number of mutations after transcription and translation in the presence of Qβ replicase (in either of the forms used).

20

Example 9

Addition of artificial Qβ sequences.

In an attempt to increase the efficiency of Qβ replicase activity, specific Qβ binding sites were added to both the 5' and 3' ends of the anti-GlyA T7-scFv

25   template by PCR. This new template (amplified with primers N5904 and N5910 [sense] and N5909 [anti-sense]; Figure 11) was used in a coupled transcription/translation reaction which included Qβ replicase as either a purified Qβ replicase protein or as a gene template under the control of a T7 transcriptional promoter which could be simultaneously synthesized during the coupled

30   transcription/translation reaction. Selections were performed using HepB, GlyA or BSA coupled magnetic beads and products recovered after RT-PCR. The presence of artificial Qβ stemloop sequences (i) did not have an adverse effect on coupled transcription, translation and selection and (ii) in most cases increased the amount of products recovered by RT-PCR after selection.

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Example 10

Replication of anti glycophorin scFv transcripts by Qb replicase in the coupled transcription/translation rabbit reticulocyte system.

The T7-1C3 and T7-4C2 scFv templates for ribosome display were
5   constructed as described in example 3 and subjected to coupled
transcription/translation, under the following conditions. Standard coupled
transcription/translation reactions were modified by the addition of $Q\beta$ replicase
(purified as detailed in example 1). In a standard 20µl reaction 1ml of 20µg/ml
enzyme was added. Previously we have compared the effect of $Q\beta$ replicase
10   concentration on replication of anti GlyA 1C3 scFv and anti Hepb 4C2 scFv in the
coupled system and observed that 1ml of this sample provided the optimum
replication. Manganese chloride was added to a final concentration of 0.5mM as
this has been shown in published reports to decrease the requirement for
transcription/translation factors. Reactions were allowed to continue for 2 hrs at
15   37°C. The replicated transcripts were analysed by RT-PCR after removing DNA
template by DNAase I digestion in 40mM Tris-HCl pH7.5, 6mM $MgCl_2$, 10mM NaCl
at 30°C for 20 mins. Standard phenol extraction was used to remove DNAaseI and
other proteins. Samples were ethanol precipitated and the RNA precipitate
dissolved in RNAase-free water. The RNA was assayed by RT-PCR using primers
20   specific for each template,see example 3, and the PCR products (DNA) compared
by agarose gel electrophoresis. The DNA bands were visualised by staining with
ethidium bromide. The agarose gel was subjected to densitometry by scanning the
digitised image with the gel-pro analyzer commercial software. Figure 13 shows the
densitometer traces of the agarose gel from which it can be seen that in the sample
25   containing the purified $Q\beta$ replicase there is an increase in the amount of template
produced.

Example 11

Replication and mutation of anti glycophorin scFv and anti Hepatitis B scFv by $Q\beta$
30   **replicase from T7 polymerase transcripts; $Q\beta$ replicase mutates transcripts during RNA dependent RNA replication.**

Coupled transcription/translation reactions as detailed in previous examples
were supplemented with $Q\beta$ replicase purified enzyme to replicate and mutate the
T7 DNA dependent RNA polymerase transcribed anti GlyA 1C3 scFv RNA.
35   Following the transcription/replication/mutation/translation incubation, the sample
was treated wth DNAaseI and this enzyme removed as detailed in example 10. The

purified RNA was then used as the template for RT-PCR reactions with anti GlyA
1C3 scFv -specific primers in the reaction as detailed in example 3. The
thermostable polymerases used in these reactions were one of the high fidelity *vent*,
pfu polymerase enzymes used in accordance with the manufacturers instructions.

5   The PCR reaction products were purified with one of the commercially available
kits as noted before and the purified DNA ligated into the commercially available
plasmid pCRscript and transformed into competent *E.coli* XL1Blue cells using
standard molecular biology techniques. The transformation reactions were plated
onto YT-agar plates containing X-gal. After overnight incubation white colonies

10   (E.coli with plasmids containing DNA inserts in the multi-cloning site) were picked
and grown overnight at 37°C in 5 ml of YT broth containing 100µg/ml ampicillin.
DNA was extracted from each of the cultures with the commercial kit (Quiagen)
according to the manufacturer's instructions. The purified DNA was analysed by
DNA sequencing: the sequencing results are displayed in Figure 16. This table

15   shows mutations in a random sample of sequences representing a minute sampling
of mutations and sequence variation in the whole Qβ replicase
replication/mutation reactions.

        ## Example 12

20   **Predicted secondary structure of template RNA**

        The RNA sequences and putative secondary structures preferred by Qβ
replicase for its RNA templates have been reported (Zamora et al., 1995). To
determine whether these or related preferential structures exist in the templates for
the continuous in vitro evolution the upstream untranslated sequences: T7

25   promoter sequences. the sequences encoding the 1C3 gene, the constant light
anchor region gene. the anti hepb 4C2 scFv gene and the IgM human constant
heavy anchor region gene were analysed with the Mfold program (Zucker et al ,
1991) and compared to the Qβ replicase preferred structures (as shown in Figure 8).
From this comparison it can be seen that the 1C3 scFv has been identified to have

30   internal RNA secondary structure mimicking the M site structure of Qβ replicase. as
does the CL anchor region and shows similarity to the preferred synthetic sequence
reported by Zamora et al., 1995. This may explain the preferred replication of the
anti GlyA 1C3 scFv CL template to that of the anti Hepb 4C2 scFVCH3 by Qβ
replicase (see example 3). Therefore the CL region gene is proposed as an anchor

35   region for displayed molecules for coupled transcription/translation display and

any mutagenesis as the RNA encoding this region promotes and enhances Qβ replicase replication and associated mutation of this region and its genetic fusions.

Example13

5 **Expression protocol for pLysN-NS5B (83kDa, pI~9.05)**

pLysN-NS5B is a bacterial (cytoplasmic) expression vector with a T7 promotor. NS5B is the non-structural HepC RNA-dependent RNA-polymerase. NS5B is fused to a LysN moiety at its N terminus which are seperated by a Gly-Ser-Gly-Ser-Gly linker. 10 His residues and followed by a Asp-Asp-Asp-Asp-Lys linker
10 (GSGSGH10D4K).

This plasmid was transformed into *E.coli* strain HMS174(DE3)pLysS and grown on 1YT/Amp$_{100 \mu g/ml}$/Chloramphenicol$_{34 \mu g/ml}$ agar plates at 37°C. A single colony was selected and cultured in an overnight broth 1YT/Amp$_{100}$ $_{\mu g/ml}$/Chloramphenicol$_{34 \mu g/ml}$) at 37°C. For expression, the overnight starter culture
15 was subcultured by dilution to an A600 = 0.1 in 1YT/Amp$_{100}$ $_{\mu g/ml}$/Chloramphenicol$_{34 \mu g/ml}$ at 37°C in 2L shake flasks at 120rpm. The culture was grown until the A600 reached 0.8-1.0 and then induced with 1mM IPTG, supplemented with Amp100 μg/ml and expression allowed to proceed at 37°C for 4-5 hours.

20 The culture was harvested and centrifuged 5000g in a prechilled rotor at 4°C. The wet weight of the harvested culture was measured and the cell pellet frozen at –80°C. Approximately 3 – 4 grams was produced (wet weight) per litre of cell culture.

25 Lysis and Purification protocol

Extraction of the HepC RdRp (NS5B) was achieved by lysing the cells followed by conventional protein chemistry techniques.

To the frozen cell pellet 5ml of Buffer C (made fresh) at 4°C was added per gram of cell pellet. The mixture was stirred at 4°C using a magnetic bead until the
30 culture was completely resuspended. The culture was then sonicated with 11 bursts each of 10 seconds with 1minute pause between each burst while continually stirring with a magnetic bead throughout the sonication process. The sonicated cells were centrifuged at 75000g at 4°C for 20 minutes and the supernatant (lysate) recovered.

35 A 30% saturation of AmSO$_4$ was added to the lysate and then centrifuged at 10000g for 15 minutes. This acted to eliminate some bacterial proteins. The pellet

was discarded and to the supernatant a 50% saturation of AmSO$_4$ was added and centrifuged and again at 10000g for 15 minutes. This acted to precipitate the NS5B from a large proportion of *E.coli* proteins. The supernatant was discard and the pellet resuspended in half the original volume with Buffer C. This was dialysed in

5   Buffer C at 4°C overnight.

An aliquot from each step was analysed on SDS PAGE to confirm partial purification of ~90kDa HepC RdRp band.

The dialysed extract was loaded onto a cation exchange column with Hyper D "S" resin pre-equilibrated with Buffer C. The column was then washed with

10   Buffer C until a stable baseline was achieved. Elution was performed with a step gradient of Buffer C with 1M NaCl. It was found that NS5B eluted at a 50% NaCl ratio corresponding to a 600mM NaCl concentration.

The eluted fractions were analysed on a 10% SDS PAGE to confirm purification. NS5B was purified by this process to over 90% homogeneity with

15   minor smaller molecular weight contaminating proteins

The purified NS5B was concentrated by 50% saturation with AmSO4 and resuspension in a volume of Buffer C (with Tris pH 7.4) sufficient to redissolve the pellet. This was then dialysed in the same buffer to eliminate the AmSO4.

This purity of the NS5B was such that further purification by size exlusion

20   chromatography on a preparative Superose 12 column in Buffer C (Tris) was not necessitated, although optional.

	Buffers (Sonication Lysis, Elution, Dialysis)	Buffer C
	50mM ***Na-PO4 pH6.8 Na2HPO4	2.32ml
25	{or substitute for Tris pH 6.8	
	NaH2PO4	2.69ml }
	100mM NaCl	0.5844g
	10% Glycerol	10ml
	10mM b-Mercaptoethanol	70µl
30	0.02% NaN3	80µl
	0.25M Sucrose	8.56g
	0.1% Detergent (β-Octyl Glucopyranoside)	0.1g
	1mM Pefa-Bloc	0.1g
	Complete™ tablets(No EDTA)	2 Tablets
35	H2O	to 100ml

32

SDS-Polyacrylamide gel electrophoresis (12.5% acrylamide) and coommassie blue staining of the proteins of the purified protein showed a single band at approximately 70kD.

The HepC RdRp (NS5B) was assayed by numerous protocols. The simplest
5   method relies on the Novagen Large Scale Transcription Kit (TB069). Modified forms of this protocol have been used successfully. This method is briefly described as follows.

A double stranded DNA template digested upstream of a T7/T3/SP6 promotor is used in the presence of a T7 DNA dependent RNA polymerase to make the RNA
10   template. HepC RdRp (NS5B) in the same cocktail then amplifies the RNA produced by the T7 polymerase.

DNA template (0.5µg/ml)	1 µl (0.5ng)
ATP(20mM)	10 µl
CTP(20mM)	10 µl
GTP(20mM)	10 µl
UTP (20mM)	10 µl
5X Transcription buffer	
(400mM HEPES pH7.5, 60mM MgCl2, 50mM NaCl)	20 µl
1M DTT (1M)	1 µl
T7 polymerase (100U/ml)	1 µl
HepC RdRp (NS5B)	x µl
Nuclease free water	y µl

This method has utilised the control DNA template in the kit as well as plasmid DNA cut upstream of the T7 promotor successfully. The quantity of DNA used has been as low as 0.1ng successfully. The quantity of T7 polymerase used has been as low as 0.1µl.
30   Interestingly, the HepC RdRp (NS5B) in these experiments has been demonstrated to possess the capacity to prime off dsDNA in the absence of oligonucleotide primers and amplify RNA.

Example 14

**Cloning of Hepatitis C RNA dependent RNA polymerase coding sequence into the
eukaryotic expression vector pCDNA3.1**

Hepatitis C RNA dependent RNA polymerase coding sequence Figure 10 was
5   cloned into the vector pCDNA3.1 (Figure 9) for expression *in situ* in the coupled
transcription/translation system and concomitant replication/mutation of target
RNA. Sequence of oligonucleotides used as primers in PCR amplification of
Hepatitis C RNA dependent RNA polymerase for cloning into the EcoRI and NotI
restriction sites in the eukaryotic expression vector pCDNA3.1:

10   5' GTGGTGGAATTCGCCGCCACCTCTATGTCGTACTCTTGGACC
     5' GCACGGGCTTGGGCGATAATCCGCCGGCGAGCTCAGATC

Hepatitis C RNA dependent RNA polymerase was cloned into the pcDNA3.1
vector (named pCDNAHEPC) with a strategy similar to that described in example 2
but using the above oligonucleotides in the PCR amplification of the Hepatitis C
15   RNA dependent RNA polymerase from the vector shown in Figure 17. The methods
used to demonstrate that the Hepatitis C RNA dependent RNA polymerase were
being synthesised *in situ* were exactly as described in example 2. The results from
the coupled reaction with the Hepatitis C RNA dependent RNA polymerase
template in pCDNAHEPC are shown in Figure 18. The results shown in this figure
20   demonstrate that Hepatitis C RNA dependent RNA polymerase produces larger
amounts of transcript (scan b) than T7 polymerase alone. Here the band has a
greater intensity and is broader than the band without Hepatitis C RNA dependent
RNA polymerase indicating the effect on the RNA.

It will be appreciated by persons skilled in the art that numerous variations
25   and/or modifications may be made to the invention as shown in the specific
embodiments without departing from the spirit or scope of the invention as broadly
described. The present embodiments are. therefore. to be considered in all respects
as illustrative and not restrictive.

34

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Claims:

1.   A method for the mutation, synthesis and selection of a protein which binds
to a target molecule, the method comprising:
5   (a)   incubating a replicable mRNA molecule encoding the protein with
ribonucleoside triphosphate precursors of RNA and an RNA-directed RNA
polymerase, wherein the RNA-directed RNA polymerase replicates the mRNA
molecule but introduces mutations thereby generating a population of mutant
mRNA molecules;
10   (b)   incubating the mutant mRNA molecules from step (a) with a translation
system under conditions which result in the synthesis of a population of mutant
proteins such that after translation, mutant proteins are linked to their encoding
mRNA molecules thereby forming a population of mutant protein/mRNA
complexes:
15   (c)   selecting one or more mutant protein/mRNA complex(es) by exposing
the population of mutant protein/mRNA complexes from step (b) to the target
molecule and recovering the mutant protein/mRNA complex(es) bound thereto: and
             (d)   optionally releasing the mRNA molecules from the complex(es).

20   2.   A method for the mutation, synthesis and selection of a protein which binds
to a target molecule, the method comprising:
             (a)   incubating a replicable mRNA molecule encoding the protein with
ribonucleoside triphosphate precursors of RNA and an RNA-directed RNA
polymerase, wherein the RNA-directed RNA polymerase replicates the mRNA
25   molecule but introduces mutations thereby generating a population of mutant
mRNA molecules:
             (b)   incubating the mutant mRNA molecules from step (a) with a translation
system under conditions which result in the synthesis of a population of mutant
proteins such that after translation, mutant proteins are linked to their encoding
30   mRNA molecules thereby forming a population of mutant protein/mRNA
complexes:
             (c)   selecting one or more mutant protein/mRNA complex(es) by exposing
the population of mutant protein/mRNA complexes from step (b) to the target
molecule:

37

       (d)   repeating steps (a) to (c) one or more times, wherein the replicable
mRNA molecule used in step (a) is the mRNA obtained from complex(es) selected
in step (c);

       (e)   recovering mutant protein complexes bound to the target molecule(s);
and

       (f)   optionally releasing or recovering the mRNA molecules from the
complex(es).

3.   A method as claimed in claim 2 in which step (d) is repeated more than
once.

4.   A method as claimed in any one of claims 1 to 3 in which the mutant
proteins are linked to their encoding mRNA molecules via ribosome complexes.

5.   A method as claimed in any one of claims 1 to 4 in which steps (a) to (d) are
carried out simultaneously in either a single or multiple chambered vessel, wherein
the multiple chambered vessel allows the transfer of fluids between chambers.

6.   A method as claimed in any one of claims 1 to 5 in which the RNA-directed
RNA polymerase

       (i)   introduces mutations into the replicated RNA molecule at a frequency
of at least one point mutation in 10^4 bases; or

       (ii)   introduces at least one insertion or deletion at a frequencey of 10^{-4}.

7.   A method as claimed in any one of claims 1 to 5 in which the RNA-directed
RNA polymerase

       (i)   introduces mutations into the replicated RNA molecule at a frequency
of at least one point mutation in 10^3 bases; or

       (ii)   introduces at least one insertion or deletion at a frequencey of 10^{-3}.

8.   A method as claimed in any one of claims 1 to 7 in which the RNA-directed
RNA polymerase is selected from the group consisting of Qβ replicase, Hepatitis C
RNA-directed RNA polymerase, Vesicular Stomatitis Virus RNA-directed RNA
polymerase. Turnip yellow mosaic virus replicase and RNA bacteriophage phi 6
RNA-dependent RNA.

9.   A method as claimed in any one of claims 1 to 8 in which the RNA-directed RNA polymerase is Qβ replicase.

10.   A method as claimed in any one of claims 1 to 9 in which the translation system is a cell-free translation system.

11.   A method as claimed in claim 10 in which the cell-free translation system comprises an S-30 extract from *Escherichia coli*.

12.   A method as claimed in claim 10 in which the cell-free translation system comprises a reticulocyte lysate.

13.   A method as claimed in any one of claims 1 to 12 in which the translation system comprises oxidised and/or reduced glutathione at a total concentration of between 0.1mM and 10mM.

14.   A method as claimed in claim 13 in which the glutathione concentration is between 2mM and 7mM.

15.   A method as claimed in claim 13 in which the translation system comprises oxidised glutathione at a concentration of about 2mM and reduced gluthatione at a concentration of between 0.5 mM and 5mM.

16.   A method as claimed in any one of claims 1 to 15 in which the replicable mRNA molecule is derived from a template selected from the group consisting of RQ135 RNA, MDV-1 RNA, microvariant RNA, nanovariant RNAs, CT-RNA and RQ120 RNA.

17.   A method as claimed in any one of claims 1 to 16 in which the replicable mRNA molecule is derived from a vector which comprises a template selected from MDV-1 RNA and RQ135 RNA.

18.   A method as claimed in any one of claims 1 to 17 which further comprises the step of transcribing a DNA template to produce the replicable mRNA.

39

19.   A method as claimed in claim 18 in which the DNA template is a linear DNA molecule.

20.   A method as claimed in claim 18 or claim 19 in which the DNA template
5   comprises:
        (i)   an untranslated region including a control element which promotes transcription of the DNA into mRNA and a ribosome binding site;
        (ii)   an open reading frame encoding the protein which binds to the target molecule: and
10   (iii)   a stemloop structure situated upstream of the open reading frame.

21.   A method as claimed in claim 20 in which the stemloop structure is a replicase binding sequence.

15   22.   A method as claimed in claim 21 in which the replicase binding sequence is between 15 to 50 nucleotides in length.

23.   A method as claimed in claim 21 in which the replicase binding sequence is between 20 and 40 nucleotides in length.
20

24.   A method as claimed in any one of claims 21 to 23 in which the replicase binding sequence is recognised by Qβ replicase.

25.   A method as claimed in any one of claims 21 to 24 in which the replicase
25   binding sequence comprises the sequence:
        GGGACACGAAAGCCCCAGGAACCUUUCG.

26.   A method as claimed in any one of claims 20 to 24 in which a second stemloop structure is included downstream of the open reading frame.
30

27.   A method as claimed in any one of claims 20 to 26 in which the ribosome binding site is derived from the MS2 virus.

28.   A method as claimed in any one of claims 20 to 27 in which a sequence
35   encoding an polypeptide is fused 3' and in frame with the open reading frame.

40

29. A method as claimed in claim 28 in which the polypeptide is an immunoglobulin constant region.

30. A method as claimed in claim 29 in which the immunoglobulin constant
5   domain is a constant light domain of the mouse antibody 1C3.

31. A method as claimed in any one of claims 1 to 30 in which the target molecule is selected from a DNA molecule, a protein. a receptor, a cell surface molecule. a metabolite. an antibody, a hormone, a bacterium or a virus.

10

32. A method as claimed in any one of claims 1 to 31 in which the target molecule is bound to a matrix.

33. A method as claimed in claim 32 in which the matrix comprises magnetic
15   beads.

34. A DNA construct comprising:
        (i)   an untranslated region including a control element which promotes transcription of the DNA into mRNA and a ribosome binding site;
20   (ii)   a cloning site located downstream of the untranslated region; and
        (iii) a replicase binding sequence located upstream of the cloning site.

35. A DNA construct as claimed in claim 34 in which the replicase binding sequence is between 15 to 50 nucleotides in length.

25

36. A DNA construct as claimed in claim 35 in which the replicase binding sequence is between 20 and 40 nucleotides in length.

37. A DNA construct as claimed in any one of claims 34 to 36 in which the
30   replicase binding sequence is recognised by Qβ replicase.

38. A DNA construct as claimed in claim 37 in which the replicase binding sequence comprises the sequence: GGGACACGAAAGCCCCAGGAACCUUUCG.

35 39. A DNA construct as claimed in any one of claims 34 to 38 in which a second replicase binding sequence is included downstream of the cloning site.

41

40. A DNA construct as claimed in any one of claims 34 to 39 in which the ribosome binding site is derived from the MS2 virus.

5   41. A DNA construct as claimed in any one of claims 34 to 40 in which a sequence encoding a polypeptide located 3' to the cloning site.

42. A DNA construct as claimed in claim 41 in which the polypeptide is an immunoglobulin constant region.

10

43. A DNA construct as claimed in claim 42 in which the immunoglobulin constant domain is a constant light domain of the mouse antibody 1C3.

44. A kit for generating a replicable mRNA transcript which comprises a DNA
15   construct as claimed in any one of claims 34 to 43.

45. A kit as claimed in claim 44 which further comprises at least one component selected from the group consisting of:

(i)   an RNA-directed RNA polymerase, preferably Qβ replicase, or a DNA
20   or RNA template coding for an RNA-directed RNA polymerase;

(ii) a cell free translation system;

(iii) a DNA directed RNA polymerase, preferably a bacteriophage polymerase;

(iv) ribonucleoside triphosphates; and

25   (v)   one or more restriction enzymes.

Affinity Maturation:
phage display and ribosome display

Figure 1

| T7 Promoter | RBS ATG | Gene of interest | Spacer |
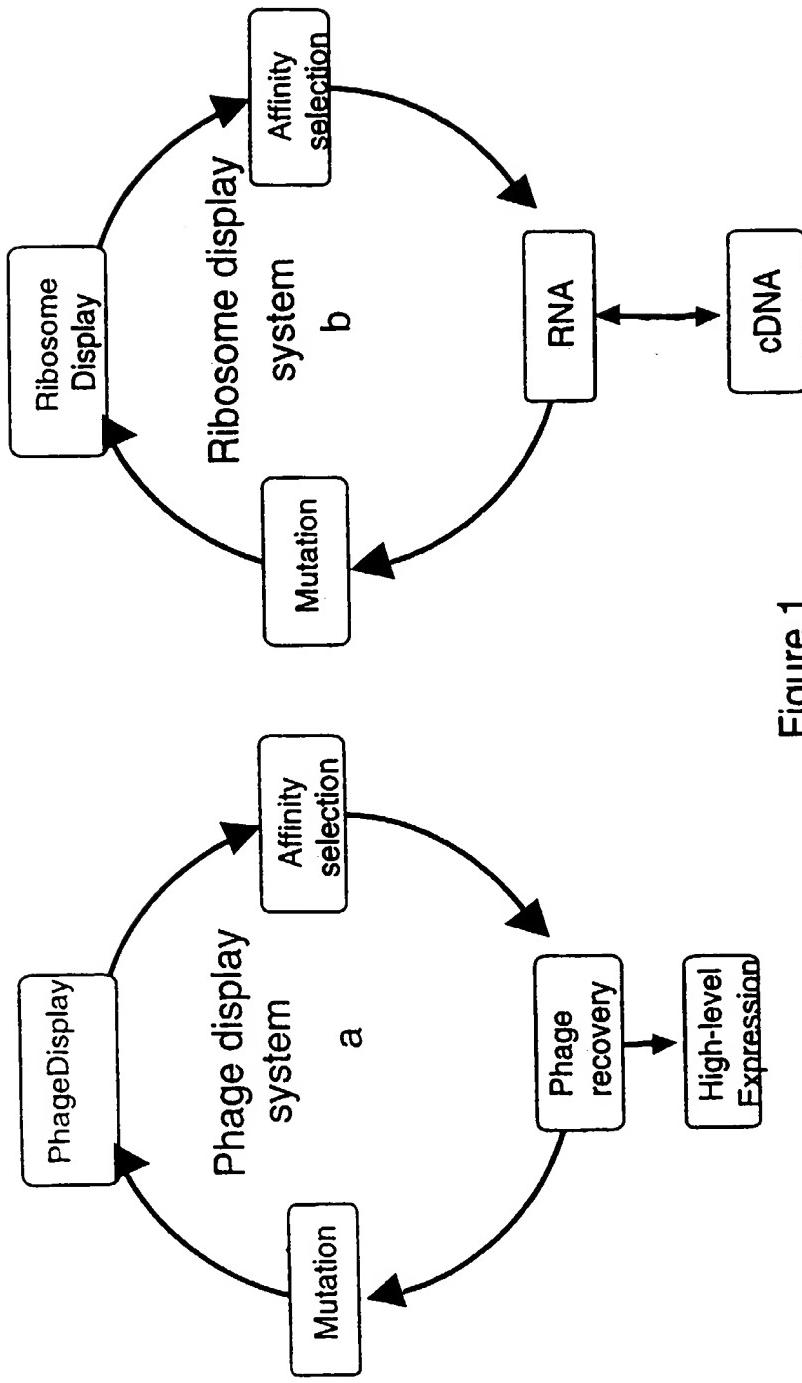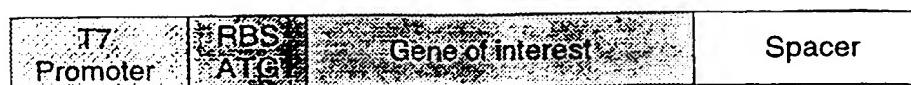
Figure 2

Figure 3: Continuous *In Vitro* Evolution
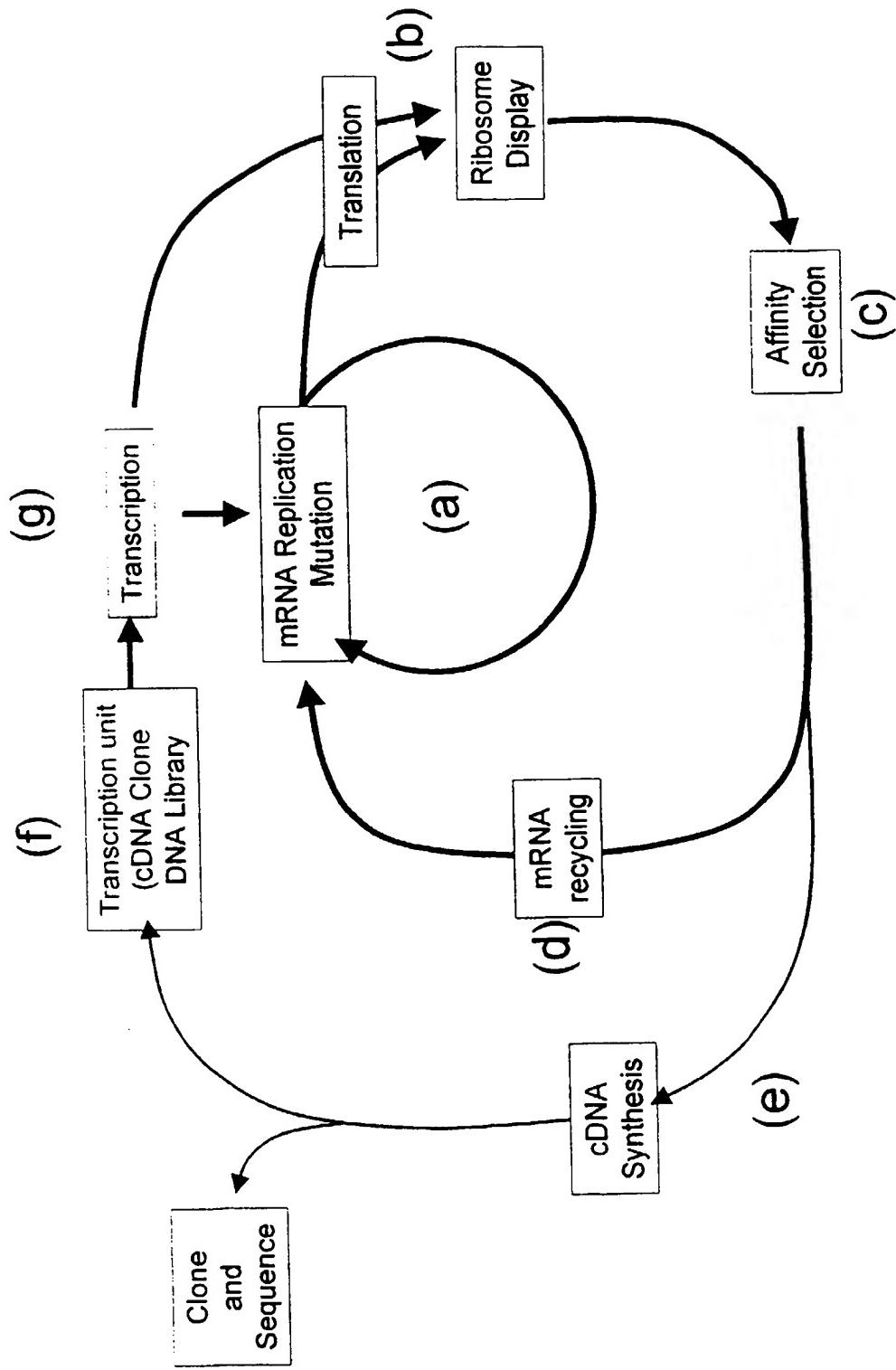
Affinity Maturation: Continuous In Vitro Evolution device

REACTION CHAMBER
Transcription/translation/binding
(Temperature controlled)

DNA

RNA to RNA* RNA*
RNA****

PROTEINS DISPLAYED

BINDING to ANTIGEN
-immobilised on
magnetic beads

Controlled
Magnet
on/off

VALVE

Rx/QB
RRL

Wash

Waste
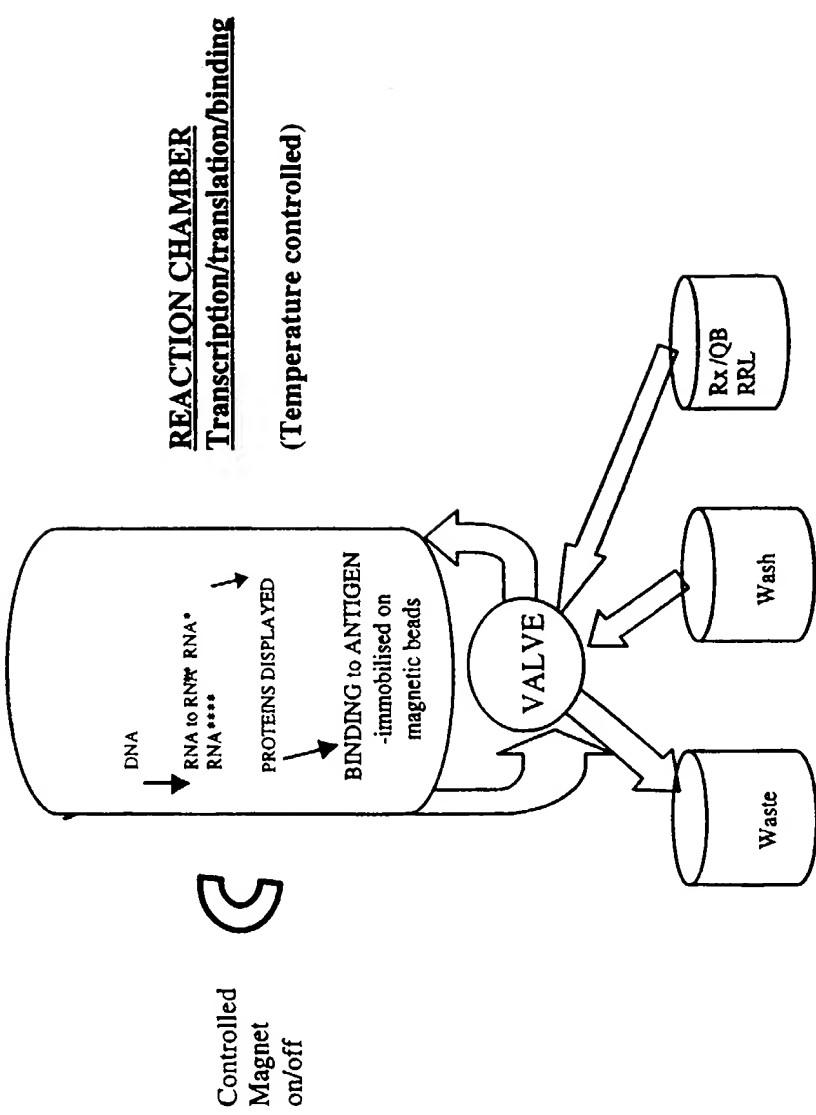
Figure 4

Figure 5

Figure 5(a) Sequence of Constant Light Region of mouse monoclonal 1C3
GCTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGA
GGTGCCTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAAAGACATCAATGTCAAGTGG
AAGATTGATGGCAGTGAACGACAAAATGGCGTCCTGAACAGTTGGACTGATCAGGACAG
CAAAGACAGCACCTACAGCATGAGCAGCACCCTCACGTTGACCAAGGACGAGTATGAAC
GACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAACTTCACCCATTGTCAAGA
GCTTCAACAGGGGAGAGTGT

Figure 5(b) Sequence of Human Constant Heavy Chain
GCAGATCAAGACACAGCCATCCGGGTCTTCGCCATCCCCCCATCCTTTGCCAGCATCTTC
CTCACCAAGTCCACCAAGTTGACCTGCCTGGTCACAGACCTGACCACCTATGACAGCGT
GACCATCTCCTGGACCCGCCAGAATGGCGAAGCTGTGAAAACCCACACCAACATCTCCG
AGAGCCACCCCAATGCCACTTTCAGCGCCGTGGGTGAGGCCAGCATCTGCGAGGATGA
CTGGAATTCCGGGGAGAGGTTCACGTGCACCGTGACCCACACAGACCTGCCCTCGCCAC
TGAAGCAGACCATCTCCCGGCCCAAGGGC

Figure 5(c) Sequence of the anti-glycophorin (1C3) scFv
ATGGCCGAGGTGAGGCTTCTTGAGTCTGGAGGTGGCCCGGTACAACCTGGAGGATCCC
TGAAACTCTCCTGTGCAGCCTCAGGATTCGATTTTAGTAGATACTGGATGAATTGGGTCC
GGCGGGCTCCAGGGAAGGGGCTAGAGTGGATTGGAGAAATTAATCAACAAAGCAGTAC
GATAAACTATTCGCCACCTCTGAAGGATAAATTCATCATCTCCAGAGACAACGCCAAAAGT
ACGCTGTACCTGCAAATGAACAAAGTGAGATCTGAGGACACAGCCCTTTATTATTGTGCA
AGACTTTCTCTTACTGCGGCAGGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACCGT
CGCCTCCGGTGGTGGTGGTTCAGGAGGAGGAGGTTCGGGTGGTGGTGGTTCGGACATC
GTCATGTCACAGTCTCCATCCTCCCTGGCTGTGTCAGTAGGAGAGAAGGTCACTATGAG
CTGCAGATCCAGTCAGAGTCTGTTCAACAGTAGAACCCGAAAGAACTACTTGACTTGGTA
CCAGCAGAAACCAGGGCAGTCTCCTAAACCGCTGATCTACTGGGCATCCACTAGGGAAT
CTGGGGTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGATTTCACTCTCACCATC
AGCAGTGTGCAGGCTGAAGACCTGGCAGATTATTACTGCAAGCAATCTTATAATCTTCGG
ACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGGG

Figure 5(d) Sequence of the anti-Hepatitis Surface antigen (4C2) scFv
CCATGGCCGATGTGAAGCTTCAGGAGTCAGGGCCTGAGCTGGTGAGGCCCGGGGTCTC
AGTGAAGATTACCTGCAAGGGTTCCGGCTACACATTCACTGATTATGCTATGCATTGGGT
GAAGCAGAGTCATGCCAAGAGTCTAGAGTGGATTGGACTTATTAGTAATTCCTTTGGTAA

SUBSTITUTE SHEET (Rule 26) (RO/AU)

TACAAACTACAACCAGAAGTTTGAGGCCAAGGCCACAATGACTGTAGACAAATCCTCCAA
CACAGGCTATTTGGAACTTGGCAGATTGACATCTGAGGATTCTGCCATCTATTACTGTGC
AAGAGTGATCGACTGGTCCTTCGATGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCT
CAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATTGTGC
TGACCCAATCTCCAGCAATCATGTTCGCATCTCCAGGGGAGAAGGTCACCATGACCTGCA
GTGCCAACTCACGTGTCAGGTACGTGCACTGGTACCAACAGAAGTCAGGCACCTCCCCC
AAAAGATGGATTTATGACACATCCAAACTGGCTTCTGGAGTCCCTGCTCGCTTCAGTGGC
AGTGGGTCTGGGACCTCTCACTCTCTCACAATCAGCAGCTTGGAGGCTGAAGATGCTGC
CACTTATTACTGCCAGCACTGGAGTAGTAACCCTCCCACGTTCGGTGCTGGGACCAAGCT
GGAAATAAAACGGGCGGCCGCAGATTATAAAGATGATGATGATAAAGCCGCGGCCCATC
ACCACCATCACCATTAAGAATTCAGCCCGCCTAATG

Figure 5 (cont'd)

7/22

Figure 6

GGGGACCCCCTTTAGGGGGTCACCTCACACAGCAGTACTTCACTGAGTATAAGAGGACA
TATGCCTAAATTACCGCGTGGTCTGCGTTTCGGAGCCGATAATGAAATTCTTAATGATTTT
CAGGAGCTCTGGTTTCCAGACCTCTTTATCGAATCTTCCGACACGCATCCGTGGTACACA
CTGAAGGGTCGTGTGTTGAACGCCCACCTTGATGATCGTCTACCTAATGTAGGCGGTCG
CCAGGTAAGGCGCACTCCACATCGCGTCACCGTTCCGATTGCCTCTTCAGGCCTTCGTC
CGGTAACAACCGTTCAGTATGATCCCGCAGCACTATCGTTCTTATTGAACGCTCGTGTTG
ACTGGGATTTCGGTAATGGCGATAGTGCGAACCTTGTCATTAATGACTTTCTGTTTCGCA
CCTTTGCACCTAAGGAGTTTGATTTTTCGAACTCCTTAGTTCCTCGTTATACTCAGGCCTT
CTCCGCGTTTAATGCCAAGTATGGCACTATGATCGGCGAAGGGCTCGAGACTATAAAATA
TCTCGGGCTTTTACTGCGCAGACTGCGTGAGGGTTACCGCGCTGTTAAGCGTGGCGATT
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Figure 6 (cont'd)

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Figure 6 (cont'd

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Figure 6 (cont'd
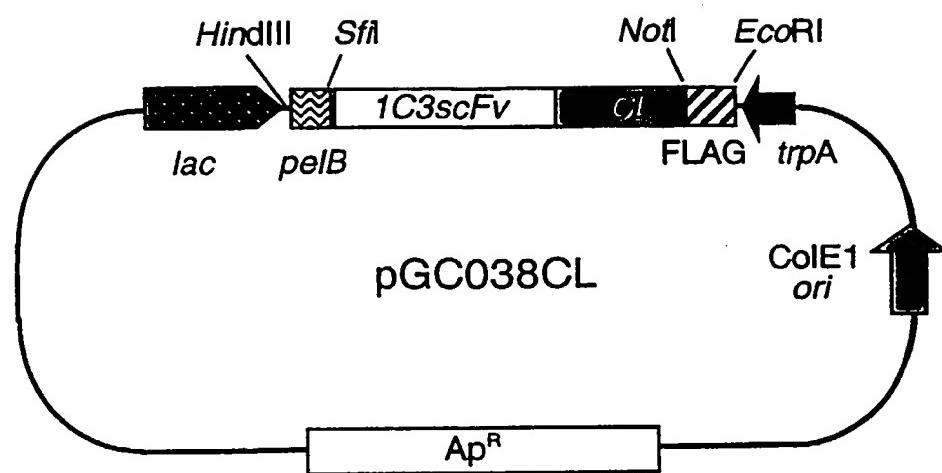
Figure 7a


Figure 7b
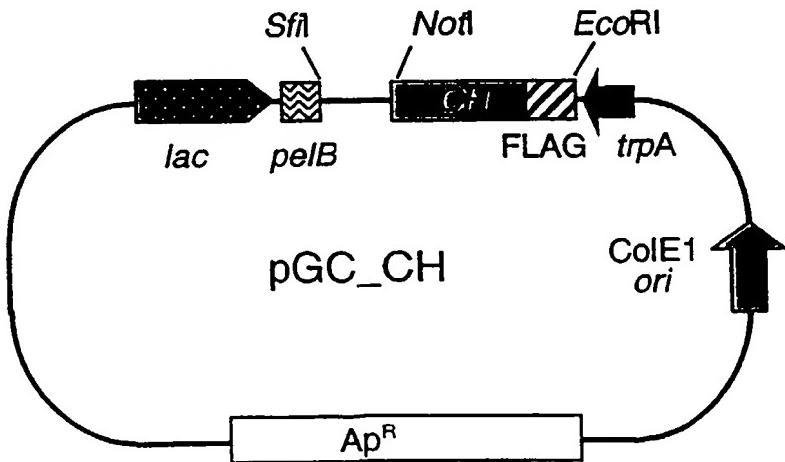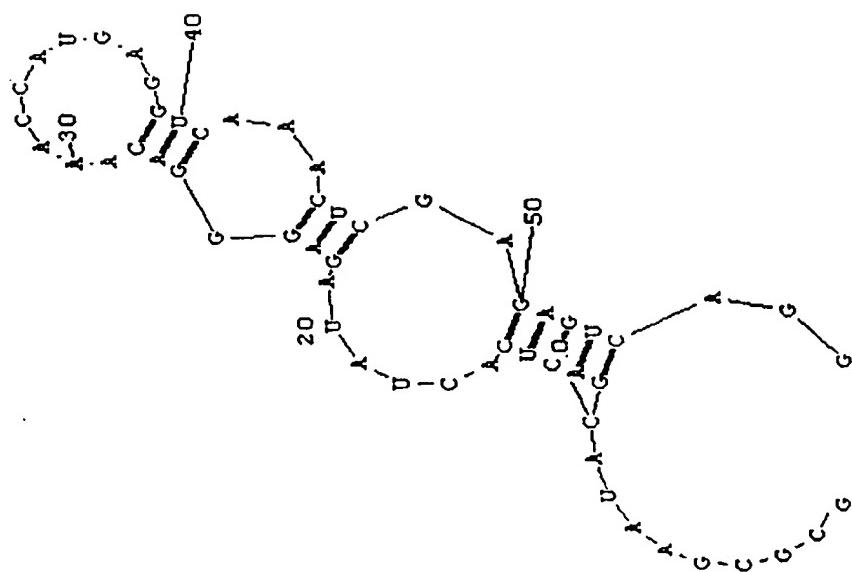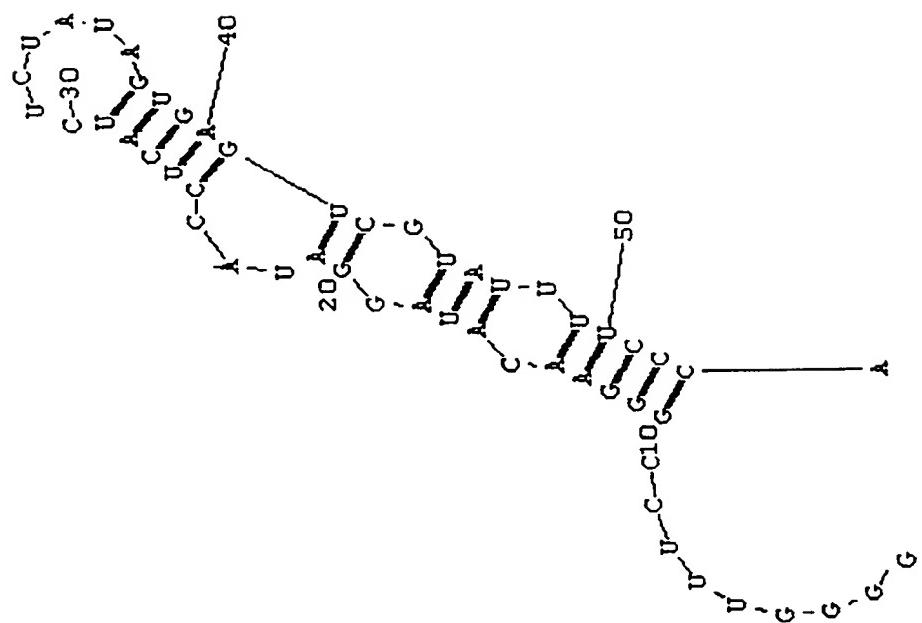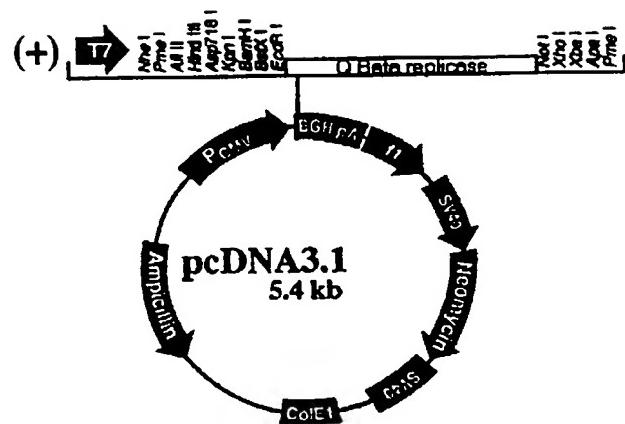
Figure 8

Figure 9

Figure 10

HCV NS5B (polymerase) sequence [1716 bases]

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Figure 11

N5266 GCG CGA ATA CGA CTC ACT ATA GAG GGA CAA ACC GCC ATG GCC GAG GTG
AGG CTT CTT GAG TCT GG

N5267 CAT CAT CAT CAT CTT TAT AAT CTG CGG CCG CAC ACT CTC CCC TGT TGA
AGC TCT TGA C

N5268 CCC CTG TTG AAG CTC TTG ACA ATG GGT GAA GTT GAT GTC TTG TGA GTG
GCC TCA CAG

N5269 CTT GTG AGT GGC CTC ACA GGT ATA GCT GTT ATG TCG TTC ATA CTC G

N5343 ACC ATG ATT ACG CCA AGC TCT AAT ACG ACT CAC TAT AGG GAA AGC TCG
CTT GTT C

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ACT TTG GCC ACC

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TGT GTG

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GGA G

N5387 CGT GAA CCT CTC CCC GGA GTT CCA GTC ATC CTC GCA GAT GCT GGC CTC
ACC

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N5659 GC AGC TAA TAC GAC TCA CTA TAG GAA CAG ACC ACC ATG GAC GTG GCC
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Figure 12

Q Beta replicase expression

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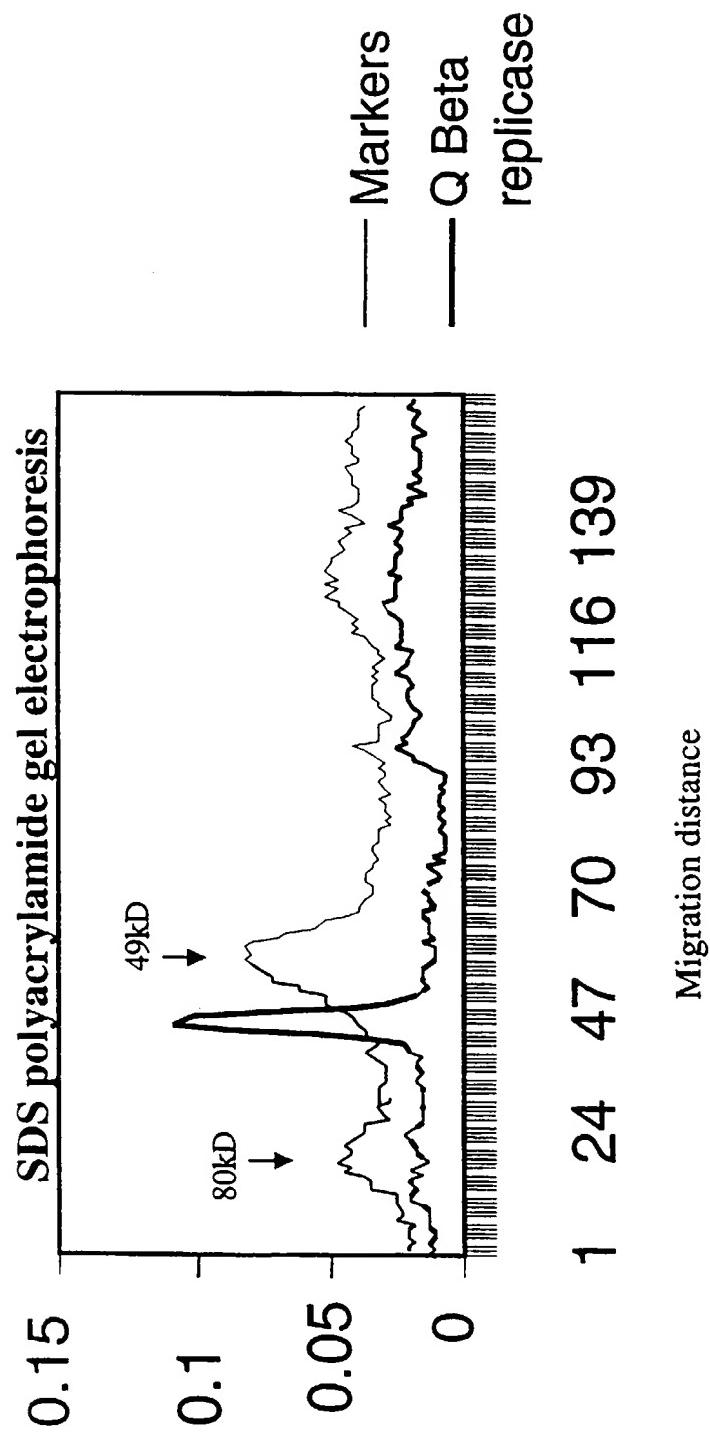
Figure 13

Translation in rabbit reticulocyte cell-free system: effect of Qβ replicase on 1C3 coupled transcription/translation

SDS polyacrylamide gel electrophoresis

80kD →

49kD →

32kD →

.... Anti GlyA scFv +Qβ replicase

——— Qβ replicase

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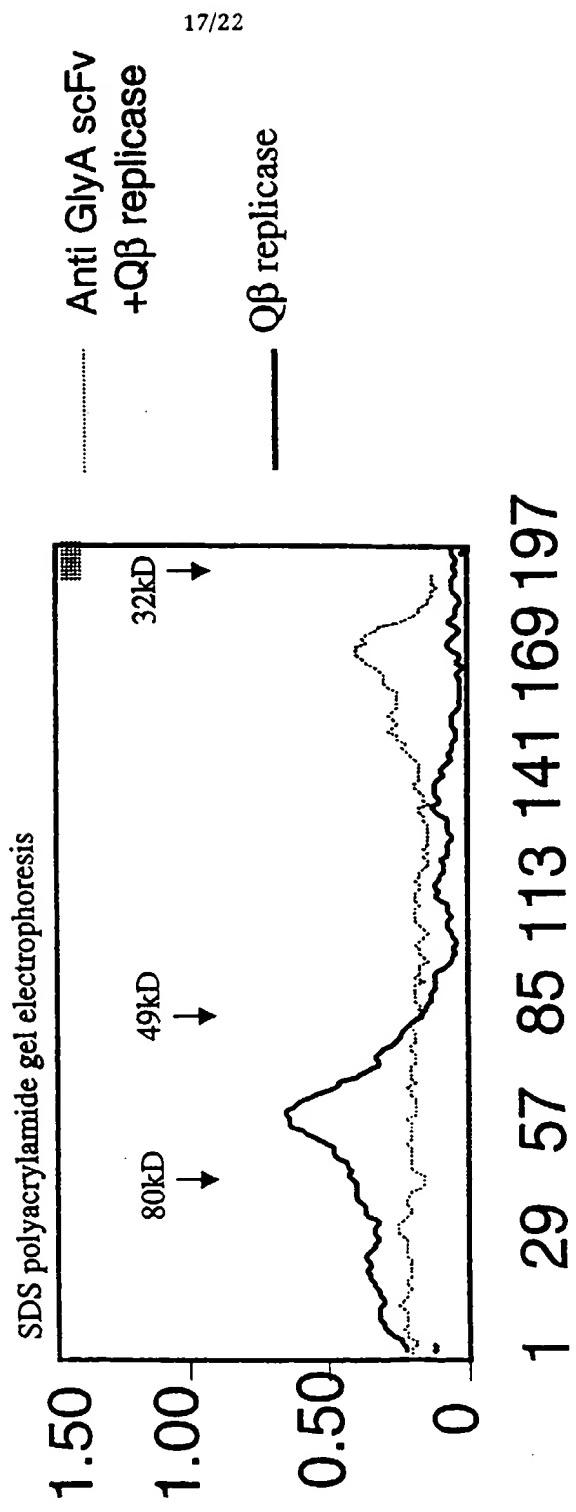
Figure 14

Sequence Name	Form of Qβ Replicase	Mutation Found
A1	Not included	None
A2	Not included	None
A3	Not included	None
A4	Not included	None
A5	Not included	T149C
A6	Not included	None
B1	Purified	A134G
B2	Purified	A279G
B3	Purified	None
B4	Purified	None
B5	Purified	T89C; G98C; A172C; T244C; A251C
B6	Purified	None
C1	pCDNAQβ	T59C; A129G; A160G
C2	pCDNAQβ	None
C3	pCDNAQβ	A30C; G82A;
C4	pCDNAQβ	None
C5	pCDNAQβ	None
C6	pCDNAQβ	A257G
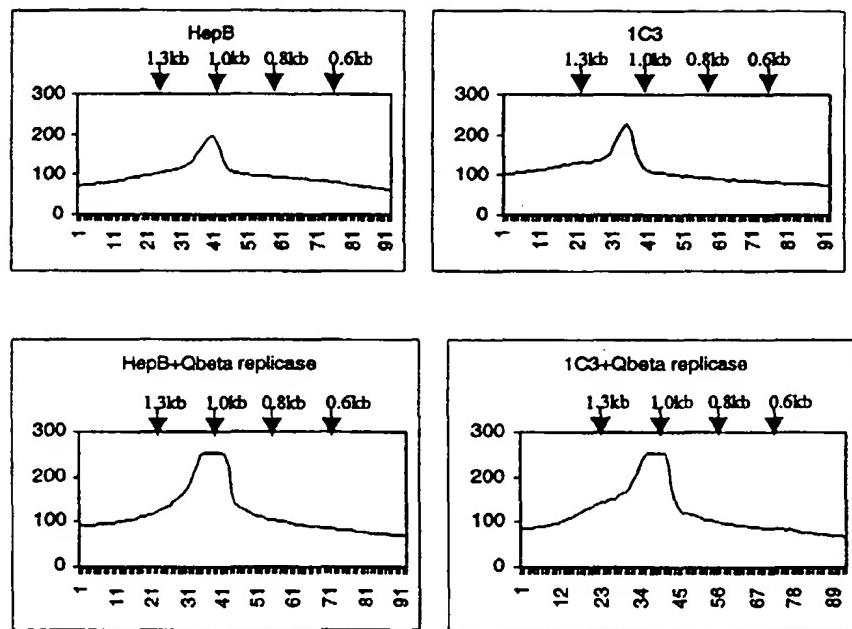
Figure 15

Figure 16

Mutation of Replicated RNA by RNA dependent Polymerases: List of
Representative Mutations Chosen at Random.

Coding region; Clone#	Replicase	Mutations
G1 (anti GlyA 1C3 scFv)	None	None
G2	Qβ purified	G124C, T234C, T329C, G367C, C379T, G385A
G3	Qβ purified	T507G, C527-delete, C543T, C557T
G4	Qβ purified	G136A, insert-141A, -164G, G216A, G330-delete, G429A, C506T, T634C
G5	Qβ purified	C208A, G210C, -211G, C213T, G320A, C403T, C597A
G6	Qβ purified	Deletion 120-441, A440G, G605A,
G7	Qβ purified	A475T, A580C,
G8	Qβ purified	A23C, G23A, G25A, G29A, G109A, G322A, C398T
G9	Qβ purified	G151T, C221-delete, C237T, C249T,
HB1 (anti Hepb 4C2 scFv)	None	None
HB2	Qβ purified	G239C, G486T, G354C,
HB3	Qβ purified	A205C, T304G
HB4	Qβ purified	A206C, G274C, T282-delete, T285-delete, G328-delete, G338–C350 substitute irrelevant sequence
HB5	Qβ purified	T397G, G398A,
HB6	Qβ purified	T561C
GC1(antiGlyA1C3 scFv)	pCDNAHepC	C57T, G221A, C557T
GC2	HepC purified	Deletion 110-162
GC3	HepC purified	Deletion 77-165
GC4	HepC purified	C353A, C366T,

Figure 17

pGE-lysN vector map, source of NS5B
(Hepatitis C RNA dependent RNA polymerase) sequence)
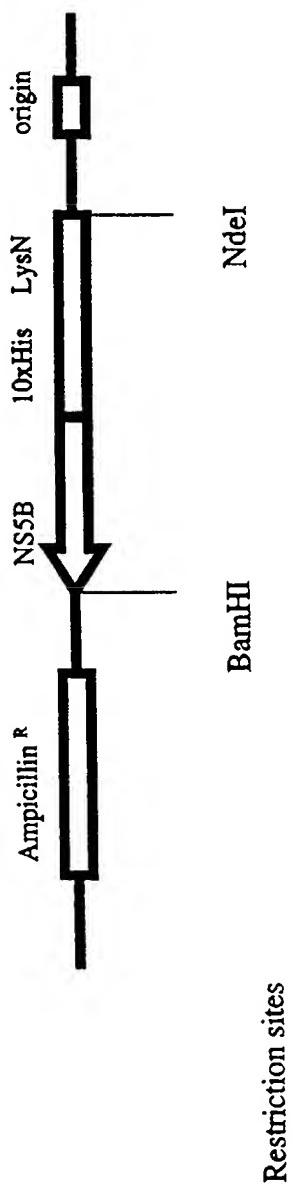
Figure 18

Effect of hepatitis C RdRP expressed in situ on 1C3 scFv RNA template in coupled reaction: RT-PCR
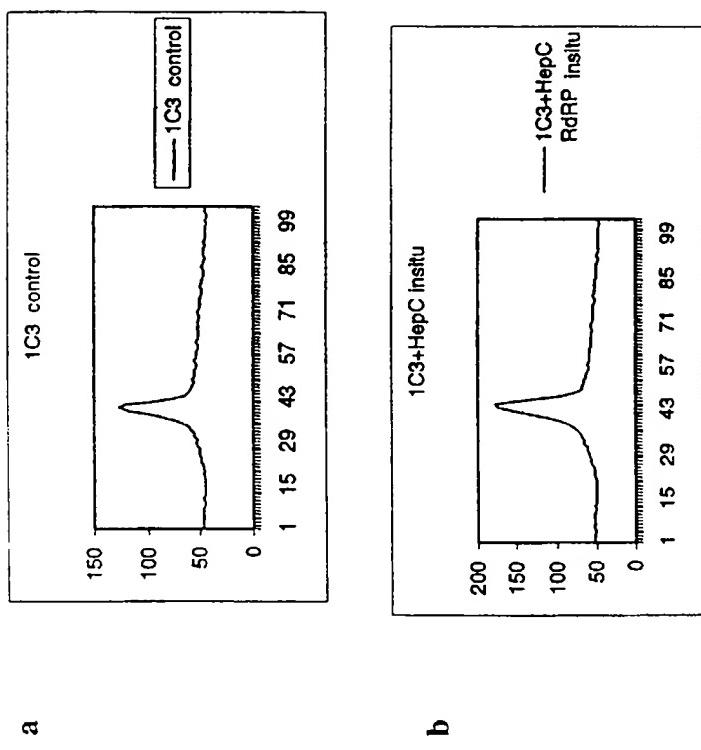
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<110> CRC for Diagnostic Technologies

<120> Continuous one step in-vitro evolution

<130> 91662

<140>
<141>

<160> 26

<170> PatentIn Ver. 2.1

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aagattgatg gcagcgaacg acaaaatggc gtcctgaaca gttggactga tcaggacagc 180
aaagacagca cctacagcat gagcagcacc ctcacgttga ccaaggacga gtatgaacga 240
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SEQ ID NO: 2
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<220>
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SEQ ID NO: 3
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      caggctgaag acctgccaga ttattactgc aagcaatctt ataatcttcg gacgttcggt 720
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 20   <213> Homo sapiens

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<220>
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      <220>
20    <223> Description of Artificial Sequence:Artificial
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<220>
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<220>
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<213> Artificial Sequence

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              primer
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        <220>
 20     <223> Description of Artificial Sequence:Artificial
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 35           primer

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 45     <220>
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              primer
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      <220>
      <223> Description of Artificial Sequence:Artificial
25          primer

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30    SEQ ID NO: 26
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35    <220>
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            primer

      <400> 26
40    gctgttatgt cgttcatact cg                                           22
```

International application No.

PCT/AU 99/00341

A.	CLASSIFICATION OF SUBJECT MATTER

Int Cl^6:   C12N 15/01   C12N 15/09   C12P 21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT, CHEM ABS SEE BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
MEDLINE SEE BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT: ((RNA dependent RNA polymerase) or (RNA directed RNA polymerase) or (RNA synthetase) or (RNA replicase) or (beta replicase)) and (mutant# or mutagen: or mutat: or amplif: or detect:) cont. on supplemental sheet.

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 602 001 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 11 February 1997 See abstract and summary of the invention.	34-40, 44, 45
Y	Proc. Natl. Acad. Sci. USA 93, pages 11558-62 (1996) Brown, D. and Gold, L. "RNA replication by Qβ replicase: A working model." See the discussion	34-40, 44, 45

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

	Special categories of cited documents:		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 May 1999	Date of mailing of the international search report **2 2 JUN 1999**
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer **TERRY MOORE** Telephone No.: (02) 6283 2569

International application No.

PCT/AU 99/00341

C (Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. Biol. Chem. 269(2), pages 1501-5 (1994) Ryabova, L. et al. "Coupled replication-translation of amplifiable messenger RNA." See the entire document.	34-40, 44, 45
X	WO A 94/06928(THE PUBLIC HEALTH RESEARCH INSTITUTE OF THE CITY OF NEW YORK) 31 March 1994 See pages 2-5.	34-40, 44, 45
X	Proc. Natl. Acad. Sci. USA 90, pages 9325-9 (1993) Morozov, I.Y. et al. "Synergism in replication and translation of messenger RNA in a cell-free system." See entire document and in particular figure 1B.	34-40, 44, 45
A	J. Mol. Biol. 249, pages 756-62 (1995) Rohde, N. et al. "The mutant distribution of an RNA species replicated by Qβ replicase." See entire document.	1-45
A	Biophys. Chem. 66, pages 179-92 (1997) Biebricher, C.K. and Gardiner, W.C. "Molecular evolution of RNA in vitro." See entire document.	1-45

Box supplemental

Continuation of Box B

CHEM ABS and MEDLINE: ((Q beta replicase) or (RNA dependent RNA polymerase) or (RNA replicase)) and (((cell free)(3n)(system) or (replication translation) or (translation transcription))

INTERNATIONAL SEARCH REPORT
Information on patent family members

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
US	5 602 001	CA	1339856	AU	73068/87	WO	8706270
		EP	346594				
WO	9406928	US	5 556 769				

END OF ANNEX